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**EXPRESSION OF APOLIPOPROTEIN B IN
HUMAN HEPATOCYTES: STUDIES ON
mRNA TRANSLATION AND
POSTTRANSLATIONAL
DEGRADATION**

by

G. Abbas Mohammadi

A Dissertation

**Submitted to the Faculty of Graduate Studies and Research
Through the Department of Chemistry and Biochemistry in
Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy at the
University of Windsor**

Windsor, Ontario, Canada

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ABSTRACT

EXPRESSION OF APOLIPOPROTEIN B IN HUMAN HEPATOCYTES: STUDIES ON mRNA TRANSLATION AND POSTTRANSLATIONAL DEGRADATION

by

Gholamabbas Mohammadi

Overproduction of apolipoprotein B100 (apoB) is a common disorder in people with coronary artery disease (CAD) and the underlying mechanisms leading to this disorder are largely unknown. Understanding the mechanisms regulating production of apoB-containing lipoproteins by hepatocytes is therefore of crucial importance. Potentially, apoB production could be regulated at different points along the pathway responsible for apoB biogenesis. These include transcription of the apoB gene, translation of apoB mRNA, translocation of the protein into the endoplasmic reticulum (ER) for the assembly of the mature very low density lipoprotein (VLDL) particles, and degradation of apoB in the ER. Regulation of apoB gene expression at the transcriptional level has already been ruled out as the levels of apoB mRNA does not change under most metabolic conditions. Posttranslational steps specially translocation of apoB into the ER and apoB degradation are under intense investigation.

The objectives of my research program were: i) to develop a mRNA-dependent cell-free system capable of translation of apoB mRNA, since common *in vitro* translation systems are unable to synthesize apoB100 because of its unusually large size; ii) to study the effect of hypolipidemic drugs including a newly developed HMG-CoA reductase inhibitor, atorvastatin, on intracellular degradation of apoB in HepG2 cells; and iii) to study degradation of different intracellular apoB pools in HepG2 cells in order to elucidate factors regulating apoB degradation.

An mRNA-dependent cell-free system was developed from HepG2 cells which can accommodate *in vitro* translation of apoB mRNA . Upon addition of cytoplasmic

RNA extracted from HepG2 cells, the HepG2 cell-free system was capable of synthesis of the full length apoB100, with a size of 550 kDa. The HepG2 cell-free system also proved to be sensitive to heterologous mRNAs and actively translated Brome Mosaic Virus RNA.

Studies on apoB degradation in HepG2 cells were also performed by using a permeabilized HepG2 system developed in our laboratory. Degradation of apoB was studied under the influence of a battery of hypolipidemic drugs including the fibrate derivatives, nicotinic acid, probucol, as well as the HMG-CoA reductase inhibitors lovastatin and atorvastatin. The fibrates, nicotinic acid, probucol, and lovastatin did not influence apoB degradation. However atorvastatin appeared to stimulate apoB degradation by approximately 25%. Atorvastatin showed this stimulatory effect under different conditions in which the HepG2 cells were provided with oleate or low density lipoprotein (LDL) as a source of lipid. Fractionation of different apoB pools in HepG2 cells showed that atorvastatin enhanced degradation of apoB bound to the ER membrane as well as apoB present in the lumen of the ER. Subsequent fractionation of luminal apoB into dense (HDL) and light (LDL-VLDL like) particles revealed that apoB in the HDL-like particles is more sensitive to the degradation under the influence of atorvastatin.

Characterization of degradation of different intracellular apoB pools in permeabilized HepG2 cells showed that degradation of membrane bound apoB occurs at a faster rate and to a larger extent compared to the luminal apoB. Degradation of apoB in the membrane pool was also not sensitive to N-acetyl-leucyl-leucyl-norleucinal (ALLN), a cysteine protease inhibitor which repressed luminal apoB degradation. This suggests that there might be two different proteolytic systems responsible for degradation of membrane bound and luminal apoB.

In summary, this study has shown that *in vitro* translation of apoB is feasible and the mRNA-dependent HepG2 cell-free system could be used as a potential tool to study mechanisms regulating apoB translation. It was also shown that the new HMG-CoA reductase inhibitor, atorvastatin, stimulates degradation of different apoB pools under various conditions which result in a decrease in the secretion of apoB by HepG2 cells. Further studies on intracellular degradation of apoB showed that degradation of

membrane bound apoB in the ER is faster compared to the degradation of luminal apoB and also is not sensitive to ALLN. Different mechanisms appear, therefore, to be responsible for degradation of apoB pools in HepG2 cells.

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DEDICATION

To: my parents, my wife, my children (Rahim, Hamid, and Roya), and my teachers

I owe to them what I have achieved

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LIST OF ABBREVIATIONS

ACAT	acyl coenzyme A:cholesterol acyltransferase
ALLN	N-acetyl-leucyl-leucyl-norleucinal
Apo	apolipoprotein
Bip	binding protein
BFA	brefeldin A
CETP	cholesterol ester transfer protein
CHD	coronary heart disease
CHO	Chinese hamster ovary
CME	cholestyramine
cpm	counts per minute
ER	endoplasmic reticulum
FCHL	familial combined hyperlipidemia
FH	familial hypercholesterolemia
FFA	free fatty acids
FLUV	fluvastatin
GTP	guanosine 5'-triphosphate
HDL	high density lipoprotein
HepG2	hepatoma G2 cells
Hep3B	hepatoma 3B cells
HMG-CoA	β-hydroxy-β-methylglutaryl coenzyme A
IDL	intermediate density lipoprotein
kb	kilobase
LCAT	lecithin-cholesterol acyl transferase
LDL	low density lipoprotein
Lp	lipoprotein
LPL	lipoprotein lipase
MDA	malondialdehyde
mRNA	messenger ribonucleic acid

MTP	microsomal triglyceride transfer protein
NADP⁺	nicotinamide adenine dinucleotide phosphate
PDI	protein disulfide isomerase
RER	rough endoplasmic reticulum
ROS	reactive oxygen species
SER	smooth endoplasmic reticulum
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SRP	signal recognition particle
TCA	trichloroacetic acid
TG	triglyceride
VLDL	very low density lipoprotein

CHAPTER 1

INTRODUCTION

1.1 Atherosclerosis

Perhaps it was the work of Carl Miler in Oslo in 1930s that shed light on the ethiology of atherosclerosis for the first time (Lusis, 1992). By showing the correlation between high plasma cholesterol, xanthomas and premature coronary heart disease (CHD) in some families, he provided evidence for the involvement of genetics in the disease as well as a link to cholesterol metabolism. It is now well known that the interaction between genetic and environmental factors leads to CHD. From epidemiological studies it has been shown that high blood cholesterol is necessary for initiation of atherosclerosis in most cases, although a number of secondary risk factors such as hypertension, diabetes, autoimmune disorders, and coagulation factor levels have also been identified. High levels of low density lipoprotein (LDL) cholesterol is now considered to be the primary risk factor for the disease because in the absence of severe or moderate hypercholesterolemia, secondary risk factors such as diabetes, hypertension, and smoking, rarely promote CHD (Lusis, 1992).

1.1.1 Pathology of Atherosclerosis

Atherosclerosis is a complex process resulting from the interaction of a number of cell types in vessels. Studies on animal models as well as examination of human vessels have shown that both in humans and in animal models atherogenesis occurs in specific sites in vessels known as areas of predilection (Berliner and Gerrity, 1992). Within a few days of feeding the animals a high cholesterol diet, massive amounts of lipid including

apolipoprotein B-containing lipoproteins accumulate in the intima of the areas of predilection (Feldman *et al.*, 1984). After about 2 weeks, monocytes start to bind to the vessel wall in the areas of predilection in these animals (Gerrity *et al.*, 1979., Gerrity RG., 1981a, Gerrity RG., 1981b). The monocytes penetrate between endothelial cells and settle in the intima where they take up lipids and form foam cells (Tsukada *et al.*, 1986). In some areas of the intima foam cells also result from binding of lipid droplets to the membrane of smooth muscle cells. Accumulation of the foam cells in the area causes the endothelium to stretch over the intima forming focal elevated areas (ChamLey *et al.*, 1977; ChamLey-Campbell *et al.*, 1981). As the number of foam cells and the thickness of the lesion increases, smooth muscle cells also spread and replicate under endothelium. forming a cap known as the fibrous cap. The smooth muscle cells may also become lipid loaded. These smooth muscle cells either arise from proliferation of preexisting intimal smooth muscle cells or migrate from media into the intima (Stary. 1990; Scott *et al.*, 1979; Thomas *et al.*, 1971). After formation of fibrous cap, monocytes continue to attach to and enter the vessel wall which results in the expansion of the lesion. As the smooth muscles form a fibrous cap over the lesion and more smooth muscle cells are formed. foam cells trapped in the core of the lesion begin to die and a large necrotic region forms. Most of the intima becomes involved in the plaque formation and fewer layers of organized smooth muscle cells remain. Lipids released from cells form vesicles and crystals of cholesterol appear at advanced stages (Bocan *et al.*, 1986). At these stages. the endothelium often breaks and thrombi are deposited onto the surface of the plaque. The thrombi later become covered with endothelium, and are incorporated into the plaque.

This increases the size of the original plaque. Repeating this procedure, forms a complicated plaque with multiple thrombi incorporated into it (Faggiotto and Ross, 1984). Since low density lipoproteins (LDL) are known to serve as the major source of cholesteryl ester deposited in atheroma (Goldstein *et al.*, 1973) lipoprotein metabolism and changes in lipoproteins that might explain the biochemical and cellular events of atherosclerosis have been heavily investigated (Mahly 1985). It is now believed that modification of LDL triggers the physiologic and pathologic functions of the participating cells which lead to atherosclerosis (Haberland and Steinbrecher, 1992)

1.2 Plasma Lipoproteins

Plasma lipoproteins are large complex particles resulting from aggregation of specific lipids and proteins. They function as vehicles to deliver water insoluble nutrients through the circulation from their site of absorption (chylomicrons) or synthesis (very low density lipoproteins) to peripheral tissues and cells that require them for anabolic and energy purposes.

There are several classes of lipoprotein particles. They are typically classified based on their electrophoretic mobility, their buoyant density, or their apolipoprotein constituent(s). The most common classification, However, is the classification based on their density. Since lipids have a lower buoyant density than water, lipoproteins float when plasma is subjected to high-speed centrifugation. Major classes of lipoproteins could be separated as distinct bands by isopycnic gradient ultracentrifugation. Based on their density, the particles are named as follows: chylomicrons ($d < 1.00$ g/mL), very low density lipoprotein (VLDL) ($d < 1.006$ g/mL), intermediate density lipoprotein (IDL)

($d=1.006-1.02$ g/mL), low density lipoprotein (LDL) ($d=1.02-1.063$ g/mL) and high density lipoprotein (HDL) ($d=1.063-1.21$ g/mL). Chylomicrons and VLDL are specialized for transport of triacylglycerols while other lipoproteins carry cholesterol and cholesteryl esters as their predominant neutral lipids (Schaefer and Levy, 1985; Stein, 1986)

1.2.1 Chylomicrons

If blood plasma taken shortly after a lipid rich meal is allowed to stand overnight, a creamy layer which is composed of chylomicrons forms at the top. Lipids consumed with food, consisting mainly of triacylglycerols and phospholipids, are hydrolyzed in the intestinal lumen and absorbed by the intestinal enterocytes. In the ER of enterocytes triacylglycerols are re-assembled again and packaged along with phospholipids, apoB48, apoAI and apoAIV into triglyceride-rich chylomicrons. These component include approximately, 82% triglyceride, 9% cholesterol, 7% phospholipid, and 2% apolipoproteins. The nascent lipoproteins then pass through the Golgi, where the apoB carbohydrate chains are processed and the particles are packed into the secretory vesicles and released into the intestinal lymph. They then enter the circulation through the thoracic duct (Imaizumi *et al.*, 1978a; Elovson *et al.*, 1988). The nascent chylomicrons are spherical particles with a diameter between 1000 to 5000 Å. In the blood stream, chylomicrons obtain additional peripheral apolipoproteins including apolipoprotein C (apo C) (Imaizumi *et al.*, 1978b) and apolipoprotein E (apoE) from the high density lipoprotein (HDL), and donate apoAIV to the HDL at the same time (Goldberg *et al.*, 1990). This is one of the multiple functions of HDL to store and exchange

apolipoproteins which are essential for hydrolysis (apoC) or receptor mediated uptake (apoE) of triglyceride rich lipoproteins.

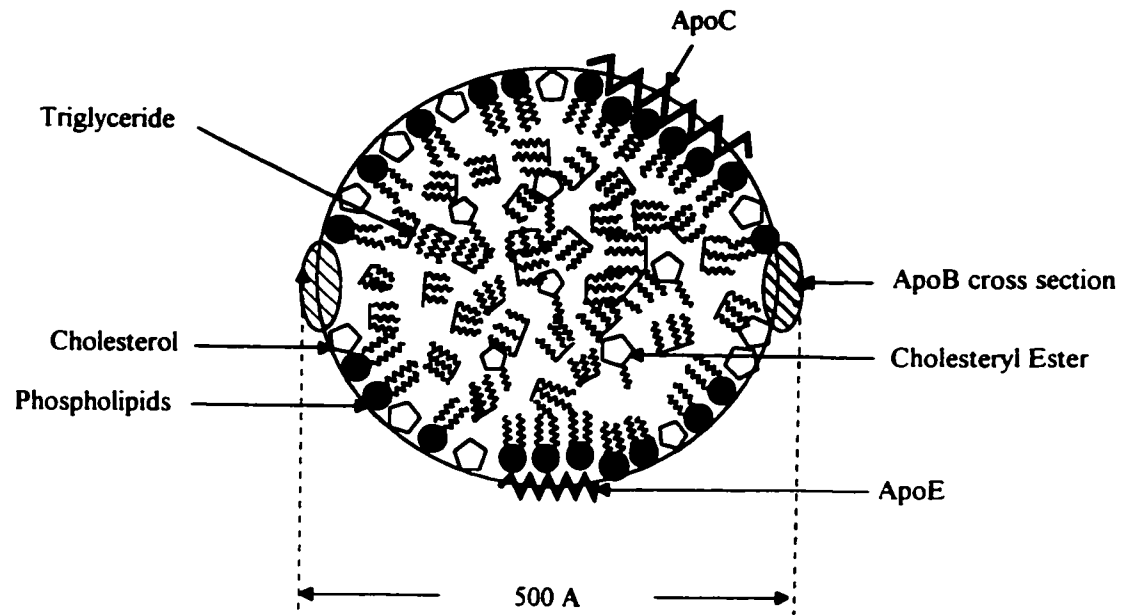
1.2.2 Very Low Density Lipoproteins

VLDL particles are composed of about 52% triglyceride, 22% cholesterol, 18% phospholipid, and 8% apolipoproteins (Figure 1.1). Like chylomicrons, the assembly of VLDL also begins with the resynthesis of triacylglycerols, which occurs in the smooth endoplasmic reticulum. However, the synthesis of VLDL takes place in hepatocytes. The nascent VLDL particles also travel through the Golgi for processing of carbohydrate chains. They are then secreted by hepatocytes into the bloodstream (Sabeson and Frase, 1977). Nascent VLDL are smaller than chylomicrons, with an average diameter less than 1000 Å, and contain a single molecule of apolipoprotein B100 (apoB100) (Elovson *et al.*, 1988). The core of VLDL particles contain mainly triacylglycerol and some cholesteryl ester. The molar ratio of cholesteryl ester:triacylglycerol in VLDL is linearly correlated with the same ratio in the cell. This indicates that the composition of VLDL core is dictated by the cellular availability of lipids (Davis *et al.*, 1982). Details of VLDL synthesis are not fully understood yet. Based on a recent study, Hamilton *et al.* (1995) proposed a two step model for apoB lipidation and VLDL synthesis. A number of studies have shown that before degradation or translocation into the ER, apoB becomes firmly associated with the membrane of the ER (Thrift *et al.*, 1992). According to the model proposed by Hamilton *et al.* (1995), the first step is the core lipidation of apoB in the RER which requires microsomal triglyceride transfer protein (MTP). At this step, apoB is released from its membrane binding site into the ER. The second step depends on the

Figure 1.1
Cross Section of VLDL Particles

Legend

The core of VLDL particles is composed of triglycerides and cholesteryl esters which are surrounded by a monolayer of phospholipids and cholesterol. Two peripheral proteins, apoE and apoC partially penetrate the monolayer. The amphipathic helix of the proteins is in contact with both fatty acyl chains and the hydrophilic phosphorylcholine. A single molecule of apoB shown as cross section in this figure, makes contact with the nonpolar core of the particle and surrounds the VLDL like a belt. [Adapted with modification from Lusis *et al.*, 1992].

Figure 1.1

synthesis of large, VLDL size TG-rich particles in the lumen of the smooth endoplasmic reticulum (SER). The synthesis of these particles can take place in the absence of apoB. The second step of apoB core lipidation and the assembly of hepatic VLDL or chylomicrons depends on the union of one apoB-rich particle built in the RER with one TG-rich apoB-deficient particle formed in the SER. The apoB-rich small particles from the RER appear to be secreted whereas the TG-rich apoB-deficient particles are not. It seems therefore that apoB contains the information required to permit the TG-rich particles to enter the secretory pathway (Hamilton *et al.*, 1995).

1.2.3 Low Density Lipoproteins

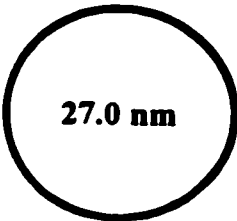


LDL is the major cholesterol carrier in the human circulation. LDL particles are mainly derived from lipolysis of VLDL by lipoprotein lipase. A spectrum of intermediate particles containing varying amounts of triglycerides are produced. These particles called intermediate density lipoproteins (IDL) could either be cleared from the circulation by the liver or they can undergo further processing to form LDL particles (Davis, 1991). The core of LDL is made of cholesteryl ester and some remaining triglyceride. This core is surrounded by a monolayer of phospholipid and cholesterol in which a single molecule of apoB100 is integrated (Elovson *et al.*, 1988). The final composition of LDL particles depends on the composition of VLDL and the intravascular change of the particle by cholesteryl ester transfer protein (CETP) coupled with lipolysis (Deckelbaum *et al.*, 1984). The complexity of these processes leads to the production of a heterogenous

spectrum of LDL particles which differ in size and lipid composition (Eisenberg *et al.*, 1984). LDL particles are distributed in plasma over a density range from 1.019 to 1.063 g/mL. Although there are as many as 15 distinct LDL subspecies present in the plasma (Chapman *et al.*, 1988), LDL particles can be classified into three major subclasses based on their physicochemical properties. These include large, intermediate and small LDL which have different ratios of cholesterol to apoB protein (Figure 1.2). Although clinical and experimental studies have shown that elevated plasma concentrations of LDL accelerate atherogenesis (Goldstein and Brown, 1977; Steinberg, 1983), it appears that the atherogenicity of LDL particles is also related to their structure, composition and metabolism as much as to their plasma concentration (Chapman *et al.*, 1995). The plasma profile of LDL particles is the result of a dynamic equilibrium between the production of VLDL particles by liver, the intravascular transformation, and the tissue catabolism of the particles (Brown *et al.*, 1981; Grundy, 1984; Havel, 1984). Recent studies have shown that small (dense) LDL particles have a lower affinity to bind to LDL receptor compared to light LDL (Nigon *et al.*, 1991) and as a result have a longer residence time in plasma (Caslake *et al.*, 1993). These dense LDL particles are therefore exposed to biological modifications which leads to their catabolism by atherogenic pathways (Steinberg *et al.*, 1989). Predominant or elevated concentrations of small dense LDL have been found in all three most common forms of atherogenic dyslipidemia namely, hypercholesterolemia, combined hyperlipidemia, and hypertriglyceridemia. The role of modified LDL in atherogenesis is discussed later in this chapter.

Figure 1.2**LDL Particle Subspecies****Legend**

LDL particles can be classified into three major subclasses on the basis of their physicochemical properties, light, intermediate, and small LDL. Chol/ApoB: number of cholesterol molecules per apoB molecule. [Adapted with modification from Chapman, 1994].

Figure 1.2

	Light	Intermeiate	Dense
			
Density	1.02-1.03	1.03-1.04	1.04-1.06
Volume $\text{nm}^3 \times 10^3$	10.3	9.8	9.2
Chol/ApoB	2750	2500	2100

1.2.4 High Density Lipoprotein

High density lipoprotein is the most abundant lipoprotein in the plasma (Stein and Stein, 1973). High density lipoproteins are a heterogeneous population of spherical and discoidal particles containing variable amounts of lipids and apolipoproteins (Gofman *et al.*, 1954). Their size varies between 70 and 100 Å. in diameter and their molecular mass between 200 and 400 kDa. Ultracentrifugation studies reveals the presence of two major subpopulations, HDL2 (d=1.063-1.125 g/mL) and HDL3 (d=1.125-1.210 g/mL). Like other classes of lipoproteins, the outer surface of the HDL particles consists of phospholipids, free cholesterol and apolipoproteins and the inner core contains triglycerides and cholesteryl esters. It has been shown that HDL2 contains 3-4 times more cholesteryl ester and triglyceride molecules than HDL3, suggesting that HDL2 is more efficient than HDL3 in fat transport (Eisenberg, 1980). During the lipolysis of triglyceride-rich lipoproteins by lipoprotein lipase, released phospholipids, free cholesterol and surface apolipoproteins are transferred to HDL3 which as a result is converted to HDL2. Conversely, hydrolysis of phospholipids and triglycerides in HDL2 by hepatic lipase and loss of most of its cholesteryl ester result in its conversion to HDL3 (Nikkila *et al.*, 1987; Nestel, 1987; Eisenberg, 1986). HDL particles may originate from larger spherical, triglyceride rich particles which are converted into discoidal HDL after depletion of their triglyceride by lipoprotein lipase and hepatic lipase (Winkler and Marsh, 1989a; Winkler and Marsh, 1989b). In an alternative pathway, they may be assembled in the circulation by association of apolipoproteins such as apoAI, apoAII, apoE and apoCs, with phospholipids derived from cell membranes and surface of

triglyceride-rich lipoproteins to form apolipoprotein-phospholipid complexes. These particles subsequently get free cholesterol from the surface of intact and lipolysed triglyceride-rich lipoproteins and are converted into discoidal HDL particles (Eisenberg, 1984). There are However, clinical conditions in which triglyceride-rich particles are not synthesized, such as abetalipoproteinemia but HDL particles are still present (Deckelbaum *et al.*, 1982). Therefore it seems likely that under normal physiological conditions, both cellular secretion and intravascular processes are involved in the production of nascent HDL. HDL function has been simply defined as a site for transient storage of potentially harmful lipids and apolipoproteins which, if not packed into lipoprotein particles, might damage cell membranes because of their potent detergent properties (Eisenberg, 1984). HDL particles play an important role in the redistribution of cholesterol between different lipoproteins as well as different tissues. By the so called reverse cholesterol transport, HDL carries cholesterol from extrahepatic tissues to the liver, where it is converted into bile acids and excreted (Eisenberg 1984; Reichl and Miller 1986; Rothblat 1986). The reverse cholesterol transport process involves (1) transfer of cholesterol from cells to a species of HDL containing phospholipids and apoAI (discoidal HDL), (2) conversion of cholesterol to cholesteryl ester on these particles by the enzyme lecithin: cholesterol acyltransferase (LCAT) which is activated by apoAI, and (3) delivery of cholesterol to the liver either directly or through its transfer to VLDL, IDL, and LDL by a reaction involving the protein cholesteryl ester transfer protein (CETP) (Miller, 1987; Gotto *et al.*, 1986). In certain animals such as the rat. HDL

delivers cholesterol to tissues which produce steroid hormones (Gwynne and Strauss, 1982).

1.3 Lipoprotein Metabolism: Chylomicrons, VLDL, and LDL

The catabolism of chylomicrons and VLDL begins in the bloodstream. They are responsible for delivering absorbed dietary fat (chylomicrons) and de novo synthesized fat (VLDL) to the extrahepatic cells. The two major lipid components of lipoproteins, triglycerides (TG) and cholesterol, have entirely different fates. TG are delivered mainly to the adipose tissue and muscle where the fatty acids are stored or oxidized for energy production. Cholesterol, in contrast is continuously shuttled between the liver, intestine, and other extrahepatic tissues. The lipoprotein metabolic pathways have been divided into exogenous and endogenous pathways, to distinguish the transport and metabolism of dietary and hepatically derived lipids, respectively. The majority of the triglyceride and some of the surface phospholipids of the lipoproteins are hydrolyzed by lipoprotein lipase (LPL) (Scow *et al.*, 1976; Eisenberg and Olivecrona, 1979). The enzyme is located on the luminal surface of the capillaries in direct contact with the blood (Olivecrona and Bengtsson-Olivecrona, 1987; Cheng *et al.*, 1981). ApoCII, transferred from HDL to the triglyceride-rich lipoproteins, binds to LPL, and serves as an essential cofactor during the hydrolysis reaction (Breckenridge *et al.*, 1978). Most of the released fatty acids are absorbed by the adjacent tissue and the rest bind to albumin (Scow *et al.*, 1976). Despite the hydrolysis of some of the phospholipid by LPL it has been suggested (Tall and Small, 1978) that some of surface phospholipid and protein accumulates during the enzymatic

removal of the core triglycerides. This monolayer is then removed perhaps by a budding process and becomes associated with HDL particles. The metabolism of this surface monolayer generated during lipid hydrolysis is one of the functions of HDL particles (Glomset and Norum, 1973; Patsch *et al.*, 1978).

After hydrolysis of the bulk of the triglycerides, the remaining particles are called chylomicron or VLDL remnants. These particles still contain a single molecule of apoB48 and apoB100, respectively, which identifies them as being originated from intestine or liver (Kane *et al.*, 1983). They also contain some apoC. and are enriched in apoE acquired from HDL. Remnant particles also obtain cholesteryl esters from HDL through a reaction catalyzed by the enzyme, cholesteryl ester:triglyceride exchange protein (CETP) (Hesler *et al.*, 1983). Removal of chylomicron remnants and a majority of the VLDL remnants by liver occurs through receptor-mediated endocytosis. Since apoB48 lacks the binding site for the LDL receptor the uptake of chylomicron remnants requires apoE (Hui *et al.*, 1984) which is also capable of binding to LDL receptor (Nagata *et al.*, 1988) . In some animals, and possibly humans, a considerable fraction of chylomicron remnants are also removed by the macrophages in the bone marrow (Hussain *et al.*, 1989a; Hussain *et al.*, 1989b). Since chylomicron remnants do not accumulate in humans with familial hypercholesterolemia (whom lack the LDL receptor). another possible candidate for the uptake of chylomicron remnants is the LDL-receptor-like protein (LPR) (Herz *et al.*, 1990) which is also the receptor of α 2-macroglobulin.

In the case of VLDL remnants, a significant fraction of particles, perhaps about 50%, are converted to LDL (Havel, 1984). During this process, VLDL remnants keep

only one molecule of apoB100 and lose most of the triglyceride, and all of the apoE and apoC proteins. It is probably the hepatic lipase that removes most of the remaining triglyceride and phospholipid, leaving the LDL highly enriched in cholesteryl ester (Demant, 1988; Auwex *et al.*, 1989).

LDL particles as the end product of VLDL catabolism in the circulation, and IDL particles are mainly catabolized via the LDL receptor. The LDL receptor is found in the liver as well as extrahepatic tissues. The receptor provides cells with cholesterol through the receptor-mediated endocytosis of both LDL and VLDL remnants (IDL). The liver removes most of the IDL and about 70% of the LDL (Spady *et al.*, 1983; Pittman *et al.*, 1982). The receptor mediated uptake of LDL is one of the best known processes of macromolecular transport across the plasma membrane of eukaryotic cells. This mechanism of transport with some variations is common between at least 25 macromolecular transport systems (Goldstein *et al.*, 1985). Studies with cultured human fibroblasts have shown there are approximately 15,000 LDL receptors on the surface of the cell. The LDL receptors are not distributed uniformly on the cells surface. They continuously move into specific areas of the plasma membrane. Although these areas make only about 2% of the cells surface, up to 80% of the receptors are clustered there. These regions have a unique feature. They can form pits of about 100-500 nm in diameter on the cell surface. Electron micrographs have shown that the pits have a fuzzy coat and they are lined on the cytoplasmic side of the membrane. In addition to the LDL receptor, each of these coated pits contains several kinds of receptors. The LDL particles, However, bind only to their own receptors as there is an extremely high affinity and

specificity between the LDL and its receptor. The coated pit then forms an endocytic coated vesicle by invagination and separation from the cell membrane. Once in the cell, the vesicle rapidly loses its coat and fuses with other uncoated endocytic vesicles. These smooth vesicles are called endosomes. The ATP-driven pump(s) then make the interior of the endosomes acidic. Due to the acidic environment, LDL dissociates from the receptor and is delivered to lysosomes. While the receptor is recycled and goes back to the cell surface, the LDL gets degraded in the lysosomes (Goldstein *et al.*, 1985; Schneider, 1989).

The cholesterol, or possibly oxidized sterols released from lysosomal hydrolysis of LDL cholesteryl esters, mediate a complex series of feedback control mechanisms to protect the cells from over-accumulation of cholesterol. First they repress the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase and HMG-CoA reductase, two key enzymes in the cholesterol synthesis pathway. Second, the cholesterol activates the enzyme acyl-CoA:cholesterol acyltransferase (ACAT), allowing the cell to store excess cholesterol in the form of cholesteryl ester droplets. Third, it inhibits the synthesis of new LDL receptors which prevents further uptake of LDL and therefore cholesterol overload (Schnider, 1991).

The plasma concentration of LDL is determined by the rate of VLDL synthesis in the liver, the number of LDL receptors, the activity of lipoprotein lipase, and possibly by other less known catabolic processes (Schnider, 1991).

An overall picture of lipoprotein metabolism is shown in Figure 1.3.

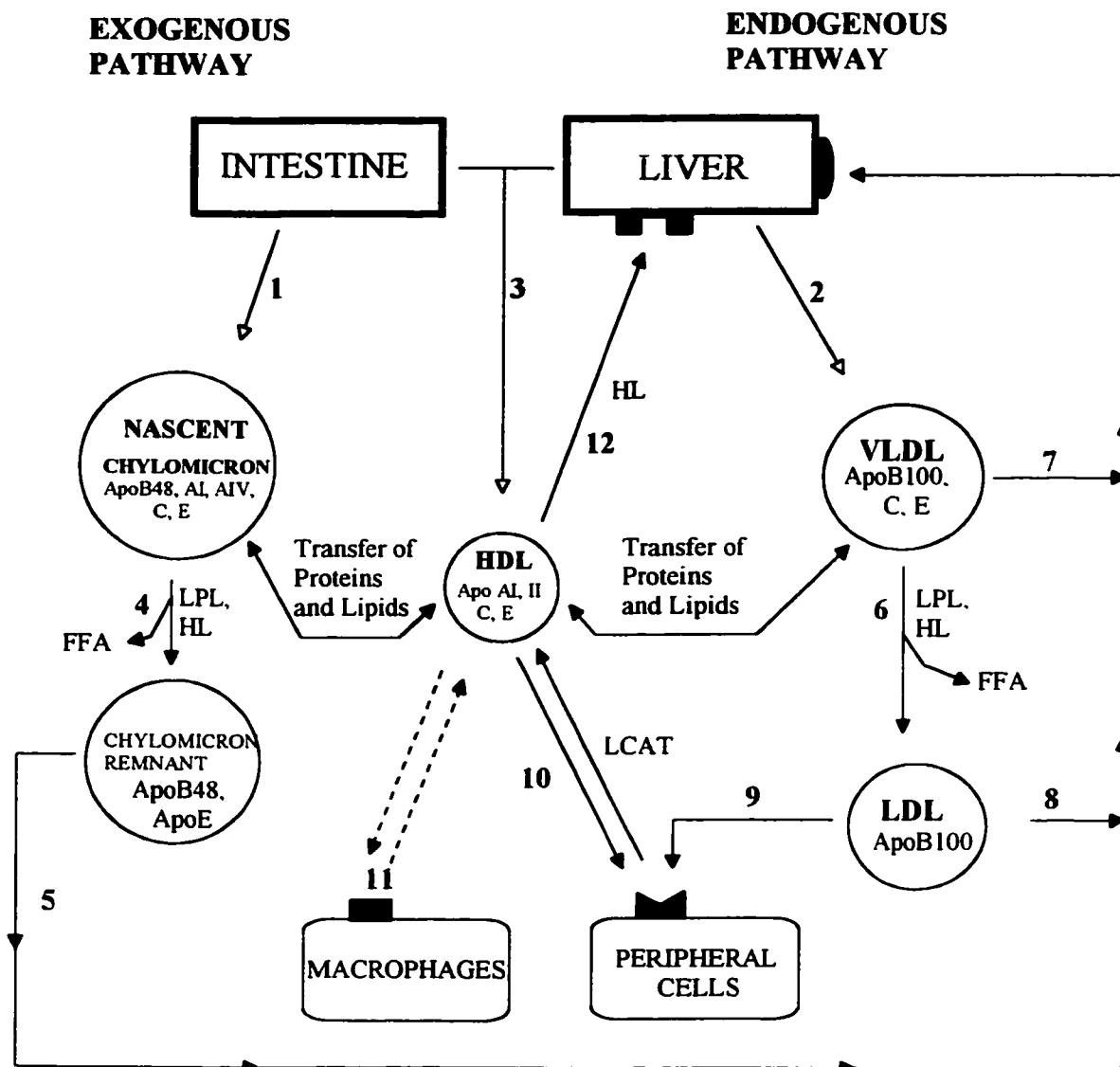
Figure 1.3**An Overview of Lipoprotein Metabolism****Legend**

Fats absorbed in the intestine are packaged into large triglyceride-rich particles called chylomicrons and are sent into the bloodstream (step 1). The chylomicrons are converted into chylomicron remnants by lipoprotein lipase (step 4). Chylomicron remnants could be removed from circulation by liver via an apoE receptor (step 5).

The liver can also synthesize and secrete triglyceride-rich particles called VLDL (step 2). Following lipolysis, VLDL particles are either converted to LDL (step 6) or removed by liver via apoE receptor (step 7). The LDL particles are mainly catabolized either by liver (step 8) or peripheral tissues (step 9) via LDL receptor. The LDL receptor recognizes both apoB100 and apoE, but not apoB48. If LDL particles are modified, they will be removed by macrophages through the scavenger receptor (step 11).

HDL particles are synthesized by both liver and intestine (step 3). As chylomicron and VLDL undergo lipolysis (steps 4 and 6), HDL pick up lipid and protein constituents from these particles. HDL pick up free cholesterol from peripheral tissues (step 10) and macrophages (step 11) and are mainly catabolized by liver. [Adapted with modification from Rifai and Warnick, 1991]

Figure 1.3



1.4 Modified Low Density Lipoproteins and Atherosclerosis

It has been known for a long time that high levels of plasma cholesterol are associated with an increased risk of atherosclerosis. The Framingham Heart study has clearly shown the positive relation between elevated plasma levels of LDL and the risk of premature coronary heart disease (Anderson *et al.*, 1987). It is believed that modification of LDL triggers changes in physiologic and/or metabolic functions of cells participating in the pathogenesis of atherosclerosis. Some of the more widely studied modifications include posttranslational, covalent modification of the apoB100, alterations in the lipid and amphiphilic components of LDL, and changes in the physicochemical structure of the lipoprotein. The potential role of modified LDL in atherogenesis dates back to the studies of Goldstein *et al.* (1979). They concluded that prolonged residence time of plasma LDL in individuals with familial hypercholesterolemia might increase lipoprotein susceptibility to modification. This disease is the result of mutations which cause loss or deficiency of functional LDL receptors (Brown and Goldstein, 1986) therefore resulting in an elevated concentrations of plasma LDL. Early development of atherosclerosis and extensively high levels of LDL are the main features of this autosomal dominant disorder (Brown and Goldstein, 1986). The absence of active LDL receptors accelerates deposition of LDL in the arterial intima. It is believed that in the arterial intima, where the residence time of LDL is relatively long and the concentration of antioxidant defense is low, the LDL oxidation occurs to a substantial extent. This oxidized LDL is believed to play a significant role in the initiation of atherosclerosis (Steinberg *et al.*, 1989). Daugherty *et al.* (1988) extracted LDL from aortas of WHHL rabbits and found several changes such

as altered electrophoretic mobility, increased fragmentation of apoB and increased malondialdehyde content, suggesting oxidation of LDL. Yla-Herttuala *et al.* (1989) isolated LDL from human atherosclerotic lesions and found that the extracted LDL had a higher density compared to plasma LDL. There was also increased electrophoretic mobility of lesion LDL, an increase in the total cholesterol:protein ratio in the particle, a moderate rise in the contents of lysophosphatidylcholine, and increased fragmentation of apoB. The apoB as well as apoB fragments in the lesion LDL showed reactivity toward antibodies against malondialdehyde-lysine and 4-hydroxynonenal-lysine adducts (Yla-Herttuala *et al.*, 1989). In the intima, the modified LDL triggers a series of events which leads to the attachment of monocytes to the endothelium and their migration into the endothelial intima. Macrophage products such as reactive oxygen species (ROS), malondialdehyde (MDA), and other aldehydes can further modify LDL to form oxidized LDL. The oxidized LDL is then recognized by the macrophage scavenger receptor, leading to cholesteryl ester accumulation in macrophages and formation of foam cells (Navab *et al.*, 1991). The progress of atherogenesis process which finally leads to the formation of atherosclerotic plaque has been described in previous sections in detail.

In the circulation, the oxidized LDL is rapidly cleared. Therefore the pathophysiologic significance of oxidized LDL in plasma is unclear (Steinbrecher *et al.*, 1987; Gorog and Kakkar, 1987).

1.5 Pharmacological Modulation of Plasma Lipoproteins

Several studies have proved that reduction of cholesterol-carrying lipoproteins in circulation can reduce the risk of myocardial infarction (Lipid Research Clinics Program,

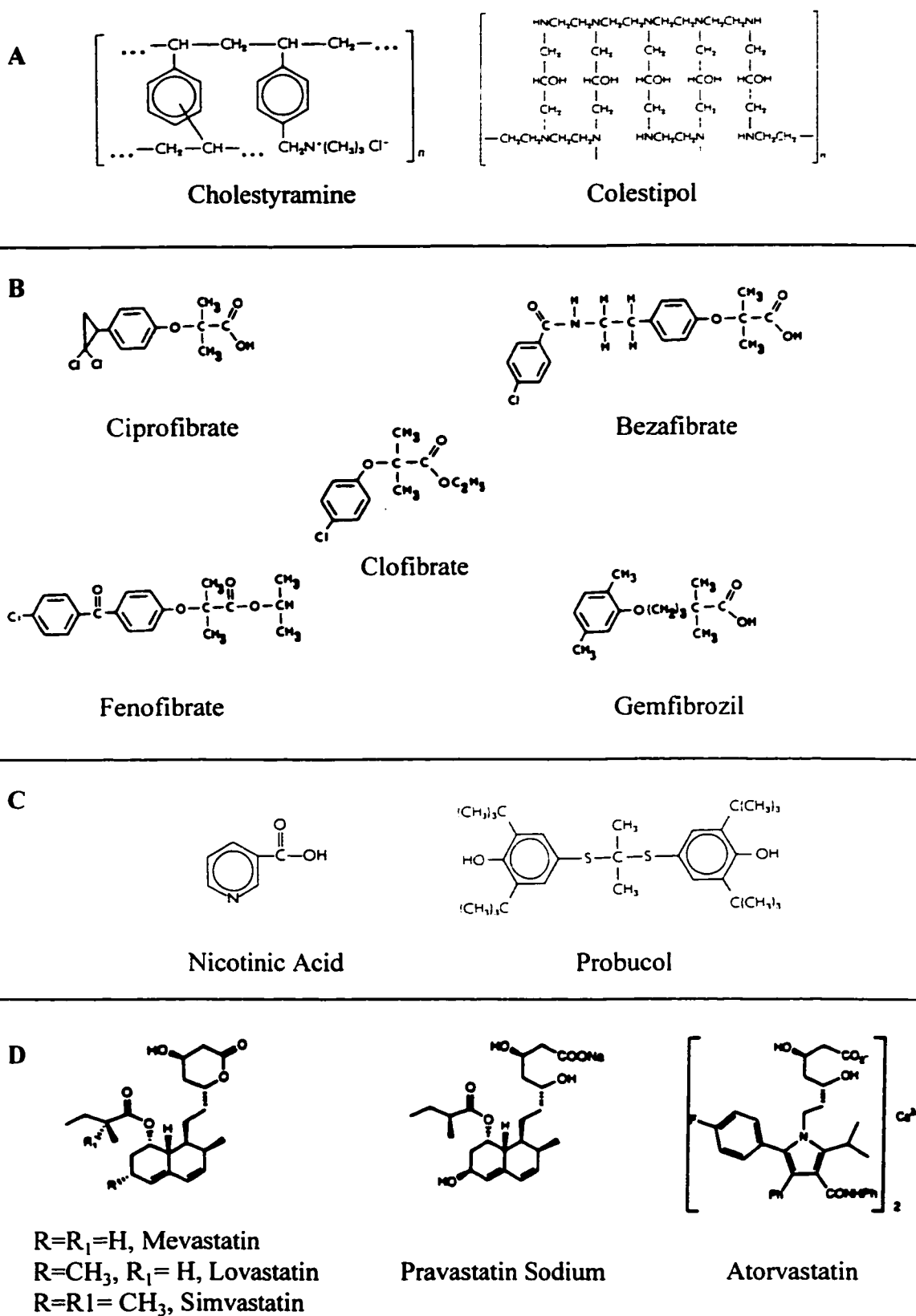
1984a, 1984b; Helsinki Heart Study, 1987). The common forms of hypercholesterolemia with a cholesterol concentration between 5.2 to 7.8 mmol/l (200-300 mg/dl) (LDL 3.5-5.5 mmol/l [135-215 mg/dl]) are usually treated by correction of overweight and use of a lipid lowering diet. Drug therapy is recommended if the patients do not respond to these measures. Different classes of hypolipidemic drugs have been used to lower the plasma concentration of cholesterol. The most commonly used agents include: 1) bile acid sequestrant resins, cholestyramine and colestipol; 2) fibric acid derivatives, bezafibrate, clofibrate, ciprofibrate, fenofibrate, and gemfibrozil; 3) nicotinic acid; 4) probucol; and 5) HMG-CoA reductase inhibitors, lovastatin, pravastatin, and simvastatin (Figure 1.4).

Two resins, cholestyramine and colestipol are members of this class which are currently in use. They have been successfully used in the treatment of heterozygous familial hypercholesterolemia and patients with common hypercholesterolemia. The drugs are taken orally by dispersing sachets containing 4-5 g of resin in fluids, 1-3 times daily. They reduce the reabsorption of bile acids. As a result increased production of bile acids depletes hepatocytes from cholesterol inducing increased LDL receptor activity. LDL catabolism is then increased and plasma cholesterol levels fall by 20-30% at full dosage (Assman, 1993). In a recent study (Bard *et al.*, 1995), effects of cholestyramine (CME) on LDL- and HDL-cholesterol, triglycerides, and apolipoproteins were compared to that of fluvastatin (FLUV). Treatment with FLUV (40 mg/d) or CME (16 g/d) for 12 weeks resulted in a significant reduction in LDL-cholesterol and a significant increase in HDL-cholesterol. Fluvastatin also reduced plasma TG levels, whereas cholestyramine

Figure 1.4**Chemical Structure of Hypolipidemic Drugs****Legend**

A) bile acid sequestrant resins, cholestyramine and colestipol; B) fibric acid derivatives, bezafibrate, clofibrate, ciprofibrate, fenofibrate, and gemfibrozil; C) nicotinic acid and probucol, D) HMG-CoA reductase inhibitors, mevastatin, lovastatin, simvastatin, pravastatin, and atorvastatin.

Figure 1.4



caused an increase in TG concentration.

Fibrates have been prescribed to treat human dyslipidemia for at least 30 years (Haubenwallner *et al.*, 1995). These drugs increase the activity of the lipoprotein lipase, therefore enhancing catabolism of VLDL triglyceride. This in turn facilitates transfer of cholesterol from VLDL to HDL. They also appear to decrease VLDL production and increase biliary excretion of cholesterol. Plasma triglyceride is therefore decreased and HDL cholesterol increased. The fibrates decrease plasma cholesterol 5-25%. Studies with transgenic mice have suggested that some fibrates could decrease lipid levels by lowering apoCIII gene expression (Haubenwallner *et al.*, 1995). It was shown in this study that bezafibrate and fenofibrate reduce plasma TG by about 50%. These drugs also reduced hepatic apoCIII mRNA and plasma apoCIII dramatically. Gemfibrozil also strongly decreased plasma TG. However, it showed an intermediate effect in reducing apoCIII mRNA and plasma apoCIII. Clofibrate on the other hand did not reduce plasma TG and only partially reduced apoCIII mRNA and plasma apoCIII. Fibrates did not decrease apoB or VLDL production, therefore it was concluded that they exert their effect by enhancing VLDL remnant metabolism (Haubenwallner *et al.*, 1995).

Nicotinic acid is also an effective lipid lowering drug which decreases VLDL and LDL production thus decreasing the TG levels and to smaller extent cholesterol concentration (Grundy *et al.*, 1981). It also increases the HDL concentration. However, its use is usually concomitant with a number of acute and chronic side-effects such as severe flushing and itching, gastrointestinal discomfort, hyperuricemia, appearance of acute gout, and a number of others. The mechanism of action(s) of nicotinic acid is not

yet known. It shows a wide range of effects including inhibition of lipolysis in adipose tissue, decreased esterification of triglycerides in the liver, and increased activity of lipoprotein lipase (Gey and Carlson, 1971).

Probucol is a potent antioxidant and it shows its effect through protection of LDL from oxidation (Noguchi *et al.*, 1994). Probucol is a moderate cholesterol lowering agent showing different responsiveness in different individuals. It decreases both LDL (5-15%) and HDL (up to 25%).

HMG-CoA reductase inhibitors (statins) are the newest class of lipid lowering drugs in use. The majority of cholesterol found in the human body is synthesized endogenously. The principal site of cholesterol synthesis is the liver and the rate limiting enzyme in the cholesterol biosynthesis pathway is HMG-CoA reductase (Bocan *et al.*, 1992). The synthesis of HMG-CoA reductase and LDL receptor are regulated coordinately (Brown and Goldstein, 1986). By inhibiting HMG-CoA reductase, statins reduce cholesterol concentration within the liver cell, which in turn causes an increase in the production of hepatic LDL receptors. This eventually results in the reduction of plasma LDL by increasing the clearance of small VLDL, IDL, and LDL (Bocan *et al.*, 1992; Gaw *et al.*, 1993). It has also been shown that in non-familial hypercholesterolemia and in familial combined hyperlipidemia, HMG-CoA reductase inhibitors can lower cholesterol concentration by inhibiting lipoprotein production (Arad *et al.*, 1992). Several inhibitors of HMG-CoA reductase have been or are being developed. They can be divided into three groups: 1) drugs extracted from fungi such as compactin, lovastatin, and

pravastatin; 2) synthetic compounds like BMY-22089, and fluvastatin; and 3) semisynthetic compounds such as simvastatin (Alberts, 1989). The cholesterol lowering effect of lovastatin, pravastatin, and simvastatin which are currently in use have been well documented. They reduce plasma cholesterol by over 30% and LDL cholesterol by about 40% (Walker, 1988; McTavish *et al.*, 1991; Todd and Goa, 1990).

Atorvastatin, a new HMG-CoA reductase inhibitor which is still under investigation for therapeutic approval, appears to be more efficient in reducing LDL-cholesterol (Black, 1995).

1.6 Apolipoprotein B100 and Atherosclerosis

It is now well known that apoB100 is necessary for the assembly and secretion of VLDL. Most forms of hypobetalipoproteinemia, a disorder characterized by low levels of triglyceride-rich lipoproteins and LDL are associated with mutations of the apoB gene. These mutations result in the expression of truncated forms of apoB, some of which are not able to assemble into mature VLDL particles (Collins *et al.*, 1988; Leppert *et al.*, 1988; Innerarity, 1990). The crucial role of apoB in the VLDL assembly could also be concluded from abetalipoproteinemia. This disease results from the deficiency of microsomal triglyceride transfer protein (MTP), which leads to the impaired assembly of apoB into the VLDL particles (Wetterau *et al.*, 1992). Overproduction of apoB-containing lipoproteins on the other hand seems to be responsible for hyperlipidemia in a large percentage of patients with a condition called hyperapoB, often characterized by high LDL-apoB but normal LDL-cholesterol (Sniderman *et al.*, 1982). Several studies

have shown that apoB concentrations are higher in patients with CHD compared to control individuals (Freedman *et al.*, 1986; Kottke *et al.*, 1986; Hamsten *et al.*, 1986). In almost all cases studied, apoB levels appeared to be a better predictor of CHD compared to total or LDL-cholesterol (Brunzell *et al.*, 1983; Boerwinkle and Utermann 1988). It is not yet understood what regulatory mechanisms control the number of apoB containing lipoproteins assembled and secreted by the liver, (Arad *et al.*, 1990). Investigation of various steps in apoB biogenesis is essential to better understand the mechanisms regulating production of apoB-containing lipoproteins.

1.6.1 Apolipoprotein B100, Structure and Function

Apolipoprotein B exists in two forms: apoB100 and apoB48. In humans, these proteins play key roles in the biosynthesis and secretion of the triglyceride-rich lipoproteins, VLDL and chylomicrons from the liver and intestine, respectively. The gene coding for human apoB (APOB) is found on the short arm of chromosome 2 (Knott *et al.*, 1985; Mehrabian *et al.*, 1986). The apoB gene is about 43 kb in size and is composed of 29 exons and 28 introns (Blackhart *et al.*, 1986). The apoB mRNA has 14121 or 14112 nucleotides depending on an insertion/deletion of 9 nucleotides in the signal sequence of the molecule (Ludwig *et al.*, 1987). Both apoB100 and apoB48 are coded by the same gene.

ApoB100 is the protein component of very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and low density lipoproteins (LDL). ApoB100 is an exceptionally large protein with an estimated molecular weight of 550,000 (Kane *et*

al., 1980). The apoB precursor has 4563 amino acids. A signal sequence of 27 residues is removed cotranslationally from this precursor giving a mature protein containing 4536 amino acids with a calculated molecular weight of 513 K. The discrepancy between the molecular weight obtained by gel electrophoresis and the calculated value is the result of glycosylation of the translation product. Since the calculated molecular weight of 513K was very close to the previous estimates of 500,00 to 550,000 daltons of protein per LDL particle, it was concluded that each LDL particle contains one molecule of apoB100. This was in agreement with the observations of Milne and Marcel (1982) that one molecule of monoclonal anti-LDL antibody binds to one particle of LDL.

ApoB48 is the N-terminal 48% of the apoB₁₀₀ and in humans it is produced exclusively in the intestine (Glikman *et al.*, 1986). In the enterocytes, through a unique mRNA editing process the nucleotide 6666 on the apoB100 mRNA is converted from C. into a U. As a result the codon 2153 for a glutamine is changed to a stop codon which results in the translation of a truncated form of apoB, namely apoB48 (Powell *et al.*, 1987; Chen *et al.*, 1987; Hospattankar *et al.*, 1987). In rats and mice the liver secretes particles containing either apoB48 or apoB100 (Kane 1983; Hardman and Kane 1986).

As opposed to other apolipoproteins which exchange between the surface of triglyceride-rich lipoprotein particles and HDL during their catabolism, apoB remains tightly bound to the original lipoprotein particle during its transport through the secretory pathway, secretion, catabolism and uptake. It is therefore considered as an integral protein (Chen *et al.*, 1986). The mature apoB However, does not contain long segments of amino

acids (19 or more) characteristic of membrane-spanning domains of integral proteins. Instead, the protein contains 39 short domains from 5 to 13 residues in length distributed throughout the length of the molecule enabling apoB to bind to the lipid ball (Olofsson *et al.*, 1987). Calculations of the composition and size of LDL, and the ratio of phospholipid, cholesterol, cholesteryl ester, and triolein show that the amount of phospholipid and cholesterol is not sufficient to cover the hydrophobic core of the particle. According to these calculations about 40% of the lipid core must be exposed to the aqueous environment or it must be covered with a protein. Since direct exposure of nonpolar lipids to water destabilizes the particle and is energetically unfavorable, it is likely that the particle is coated with a protein. According to a model proposed based on electronmicrographs, apoB forms a belt about 700 Å long, 55 Å wide, and 17 Å deep around the LDL particle (Chatterton *et al.*, 1991). The outer surface of the belt, composed of polar residues, would contact the surrounding aqueous phase. Apart from its requirement for assembly and secretion of lipoproteins and facilitating their transport in the circulation, apoB100 also contains the LDL-receptor binding domain, located approximately in the region between amino acids 3100 and 3600. This site is essential for the uptake of IDL, LDL, and possibly some VLDL by tissues (Knott *et al.*, 1986; Yang *et al.*, 1986). In a recent study Choi *et al.* (1995) proposed that the N-terminal region of apoB might contain the binding site for lipoprotein lipase (LPL) which facilitates the binding of LPL to the circulating lipoproteins (Choi *et al.*, 1995).

1.6.2 Role of ApoB in Atherogenesis: Relationship Between Plasma ApoB and CHD Risk

Hyperapobetalipoproteinemia, or hyperapoB, is a common disorder in CAD. It is characterized by an increased number of small, dense LDL particles. The plasma TG level under this condition may be normal or elevated (Kwiterovitch, 1993). Many patients show a normal or near normal LDL-cholesterol concentration. Therefore they may be better distinguished with levels of LDL-apoB instead of lipids (Sniderman *et al.*, 1982). HyperapoB might be produced for a number of reasons. These include: overproduction of VLDL by the liver (Kwiterovitch, 1993), impaired clearance of postprandial triglyceride-rich particles which involves a defective lipoprotein lipase (Nevin *et al.*, 1994) or the defective transfer of its cofactor apoCII from HDL to chylomicrons, perhaps because of an abnormal apoA-IV (Wojciechowski AP *et al.*, 1991); the antilipolytic effects of an increased level of apoCIII (Wojciechowski AP *et al.*, 1991), or finally as suggested by Sniderman *et al.* (1992), hyperapoB may be the result of reduced uptake of free fatty acids (FFA) released from hydrolysis of TG by adipocytes. This could occur because of a lack of cell's response to the stimulation of intracellular TG synthesis by the acylation stimulatory protein (ASP) (Cianflone *et al.*, 1989), also called basic protein I or BP I. If the rate of FFA uptake by adipose tissue is reduced, it will result in an increased flux of FFA to liver, leading to overproduction of VLDL apoB. Both *in vivo* and *in vitro* studies have proved that in hyperapoB the LDL-receptor activity is normal (Kwiterovitch, 1993). However, the affinity of small, dense LDL particles for the LDL receptor is decreased (Kwiterovitch, 1988; Galeano *et al.*, 1994). Because of depletion of fat-soluble

antioxidants in its core, small, dense LDL may be more susceptible to oxidation (Kwiterovitch, 1993).

Since overproduction of apoB-containing lipoproteins may be the cause of hyperlipidemia in a large number of patients (Janus *et al.*, 1980; Kissebah *et al.*, 1981; Teng *et al.*, 1986; Arad *et al.*, 1990) understanding the mechanisms involved in the hepatic assembly and secretion of apoB-containing lipoproteins is of great importance. This could potentially lead to the development of therapeutic measures which may bring overproduction of apoB and as result a considerable percentage of coronary artery disease under control.

1.7 Acute Modulation of Hepatic ApoB Production

Numerous studies support the concept that hepatic apoB secretion is metabolically regulated. Hepatic production rate of apoB-containing lipoproteins can be regulated by diet (Ginsberg *et al.*, 1985; Turner *et al.*, 1981; Grundy and Vega, 1985; Arad *et al.*, 1990), and is often increased in patients with elevated plasma levels of apoB-containing lipoproteins (Janus *et al.*, 1980). The formation of apoB100-containing lipoproteins appears to be extremely complex, and requires the coordinated synthesis of apoB, triglycerides, cholesteryl esters, phospholipids, and other components and their intracellular assembly. The specific mechanisms involved in the regulation of hepatic apoB production and faulty overproduction are under intensive investigation and are being unraveled (Sniderman and Cianflone, 1993). The potential regulatory levels of apoB production include: apoB gene transcription, mRNA translation, protein

translocation across the ER membrane, and intracellular degradation of newly synthesized apoB (Yao and McLoed, 1994).

1.7.1 Transcriptional Control of ApoB Gene Expression

Expression of apoB gene is tissue specific, and occurs only in the hepatocytes and absorptive enterocytes (Knott *et al.*, 1985), cells that are able to obtain and refine large quantities of neutral lipids. Research has shown that the interaction between *trans*-acting protein factors and *cis*-acting regulatory sequences, i.e., promoters and enhancers, regulate transcription of apoB gene. The regulatory sequences of the apoB gene have been found both in the 5' upstream region of the gene and also in the intron regions of the gene. It is believed that the expression of the apoB gene may be controlled by the interaction of multiple protein factors with the proximal promoter. Some of these factors include: apolipoprotein factor-type 1 (AF-1), nuclear factor NF-BA1, liver and intestinal transcription factor (LIT-1), and the liver specific transcription factor C/EBP (Metzger *et al.*, 1990; Kardassis *et al.*, 1992; Carlsson *et al.*, 1990). Two enhancer intronic sequences, located in the second and third introns have also been identified which play an important role in the regulation of apoB gene transcription (Brooks and levy-Wilson, 1992; Levy-Wilson *et al.*, 1992). Negative regulatory sequences have also been found to play an important role in the regulation of the apoB gene. One of these reducer elements is able to suppress apoB transcription by 50% in hepatic (HepG2, Hep3B) and intestinal (CaCo-2) cells (Paulweber 1991). Some of the members of the steroid hormone receptor superfamily, such as EAR-2 and EAR-3 have also been found to suppress apoB gene

transcription (Ladías *et al.*, 1992). It is believed that the competition between positive and negative regulatory factors for binding to the promoter region determines the level of apoB gene expression (Ladías *et al.*, 1992; Metzger, 1993). Another factor which might also play an important role in the regulation of apoB gene expression is the shape of chromatin which can control the accessibility of the promoter region to the transcriptional machinery (Levy-Wilson and Fortier, 1989). Although there are few reports suggesting that transcription of apoB gene could be modulated by factors such as exogenous VLDL (Wu *et al.*, 1994), 25-hydroxycholesterol (Dashti, 1992), or amino acids (Zhang *et al.*, 1993), the apoB mRNA levels do not change acutely under most metabolic conditions (Dashti N, *et al.*, 1989; Pullinger *et al.*, 1989; Moberly *et al.*, 1990; Kaptein *et al.*, 1991). These observations have focused attention on the co- and posttranslational regulation of apoB gene expression.

1.7.2 Translational Regulation of ApoB Gene Expression

Several studies have suggested that regulation of apoB gene expression could occur at the translational level. Work in our laboratory has shown that in HepG2 cells, thyroid hormone stimulates translation of apoB mRNA (Theriault *et al.*, 1992a), while insulin shows a suppressing effect (Theriault *et al.*, 1992b; Adeli and Theriault 1992). Insulin showed a similar effect on apoB mRNA translation in rat hepatocytes (Sparks and Sparks, 1990). Treatment of primary rat hepatocytes with dexamethasone also resulted in 50% more incorporation of [³⁵S]methionine into apoB100 without changing the mRNA levels (Wang *et al.*, 1995). Impaired translation of apoB mRNA has been suggested as the

major factor reducing hepatic production and secretion of apoB in streptozotocin-induced diabetic rats (Sparks *et al.*, 1992).

A potential mechanism for translational regulation of apoB gene might be in the formation of the polysomal complex between apoB mRNA and ribosomes. A recent study shows that polysomes containing apoB mRNA have unusual sedimentation characteristics. Even though the majority of apoB mRNA is complexed with ribosomes, the complex shows properties similar to nonpolysomal mRNA-protein complexes (Chen *et al.*, 1993). Factors such as hormones which affect translational efficiency of apoB mRNA might exert their effect through this complex. The biological significance of this phenomenon and its role in regulation of apoB mRNA is not yet known and it remains to be elucidated in future studies.

1.7.3 Posttranslational Regulation of ApoB Gene Expression

1.7.3.1 Translocation of Nascent ApoB Polypeptide Chains into the ER

Results obtained so far from investigations on apoB production suggest that translocation of newly synthesized apoB into the ER lumen may be the most important step regulating apoB production (Yao and McLeod, 1994). The general mechanism of protein secretion was elucidated through the work of Palade *et al.* (1975). More details of this process have been explained recently (Walter and Lingappa, 1986; Walter and Johnson, 1994). The process starts with translation of the mRNA coding for a secretory or a membrane bound protein. As soon as the signal sequence emerges from the ribosome, the signal recognition particle (SRP) binds to the signal sequence and slows down the

translation rate by interacting with the ribosome. The complex of ribosome, nascent protein, and SRP is then directed to the SRP receptor, an integral protein of the ER membrane. Upon binding of SRP to its receptor, through a GTP dependent reaction, the inhibition of translation is lifted and translation and co-translational transport of the nascent protein into the ER continues. Once in the ER, the protein undergoes several posttranslational modifications such as glycosylation, disulfide bond formation, folding, and subunit assembly (Ng and Walter, 1994). Extensive studies on the assembly and secretion of apoB100 containing lipoproteins, have shown that apoB binds cotranslationally or shortly after translation to the endoplasmic reticulum membrane (Bostrom *et al.*, 1986; Bostrom *et al.*, 1988; Boren *et al.*, 1990; Bamberger and Lane, 1988; Davis *et al.*, 1990). After a 3-minute pulse period, Bostrom *et al.* (1988) found that apoB remained associated with the ER membrane for approximately 15-17 minutes before it was translocated into the ER lumen. By adding exogenous trypsin, Davis *et al.* (1990) found about half of the apoB was exposed to cytosolic side of rough endoplasmic reticulum. Earlier *in vitro* studies on apoB15 has shown that the nascent apoB is translocated into the ER in a pause transfer manner (Chuck *et al.*, 1990). A 33-residue segment of apoB (residues 82-114) was later found to cause the stepwise translocation of apoB. This sequence called the 'pause transfer' sequence was also found to confer the translocation pause to heterologous chimeric proteins as well (Chuck and Lingappa, 1992). Subsequent studies showed that a ten residue sequence in the pause transfer sequence is particularly important for pausing the translocation. Analysis of the apoB molecule revealed that there are 41 candidate pause sequences throughout the apoB, six

of them present in apoB15 (Chuck and Lingappa, 1993). By using epitope-specific antibodies against apoB, Du *et al.* (1994) have shown that in Chinese hamster ovary (CHO) and HepG2 cells, apoB53 and apoB100 undertake a trans-membrane orientation so that a 69 kDa portion of the N-terminus of the protein is located in the lumen and the remaining C terminus portion of the protein resides on the cytoplasmic side of the ER membrane. Proteolytic cleavage of the arrested protein by an ALLN inhibitable system produces an 85 kDa fragment that continues translocation and is secreted. Since apoB lacks the conventional membrane spanning domains, they have suggested (Du *et al.*, 1994) that the arrest of apoB translocation across the membrane could occur in two possible ways. One is through the interaction of several hydrophobic lipid binding domains present throughout the apoB molecule with the membrane bilayer, blocking further movement of the molecule or alternatively, the C terminus of the molecule which is not translocated can inhibit translocation of the protein by folding. Translocation of the C-terminus may require cell specific factors such as microsomal triglyceride transfer protein which is missing in CHO cells. Zhou *et al.* (1995) have recently shown that the cytosolic chaperone, heat shock protein hsp70 binds to those segments of apoB exposed to the cytosol during the translation/translocation process, keeping the apoB in the proper conformation until translocation is completed. The binding of hsp70 to apoB is decreased upon treatment with oleate and increased in the presence of N-acetyl leucyl leucyl norleucinal (ALLN). This study and a number of others (Dixon *et al.*, 1991; White *et al.*, 1992) have shown that oleate increases both translocation and secretion of apoB. Recent work in our laboratory has also shown that the rate of apoB translocation into the ER is

decided by both lipid availability as well as apoB conformation (Macri and Adeli, 1996). The unusual transient arrest during translocation of apoB is believed to cause the entrance of the protein into an intracellular degradative pathway which regulates its secretion (Du *et al.*, 1994; Ginsberg, 1995).

1.7.3.2 Posttranslational Degradation of ApoB

Intracellular degradation of apoB was first noticed by Borchardt and Davis (1987). In their studies on rat hepatocytes they found that only 36% and 60% of newly synthesized apoB100 and apoB48, respectively, were secreted in the culture medium. Degradation of apoB has also been demonstrated in HepG2 cells (Sato *et al.*, 1990; Dixon *et al.*, 1991). Studies in HepG2 cells (Bostrom *et al.*, 1988; Dixon *et al.*, 1991) showed that oleate could increase the intracellular pool of apoB that was secretion competent. It was concluded that oleate exerted its effect through decreasing the extent of intracellular apoB degradation (Dixon *et al.*, 1991). Similar results were also observed in McArdle-RH 7777 cells (White *et al.*, 1992). By using cDNA constructs coding for apoBs of varying size, they showed that oleate treatment only protected apoB molecules which were long enough to form buoyant lipoproteins. These observations showed that an intracellular pathway may be responsible for degradation of apoB that fails to assemble into lipoprotein particles properly. The major site of this degradation seems to be the endoplasmic reticulum or a closely-associated compartment (Davis *et al.*, 1990; Furukawa *et al.*, 1992; Adeli, 1994). The proteolytic fragments of apoB were found in rough and smooth ER, but not in the Golgi (Davis *et al.*, 1989), and degradation is not inhibited by brefeldin A (BFA), a strong inhibitor of protein transport from ER to Golgi

(Sato *et al.*, 1990). Some studies in rat hepatocytes have also suggested post-ER degradation of apoB (Wang *et al.*, 1995; Sparks and Sparks, 1993; Verkade *et al.*, 1993). A compartment that is closely associated with ER would fit with recent data showing that other proteins (Raposo *et al.*, 1995) are sorted to degradation in a pre-Golgi intermediate tubular compartment. The involvement of lysosomal proteases in apoB degradation has also been ruled out by failure of protease inhibitors such as leupeptin, pepstatin, and chymostatin or inhibitors of lysosomal function such as chloroquine to inhibit apoB degradation (Sato *et al.*, 1990). Boren *et al.* (1990, 1992) have shown that the apoB integrated into the ER membrane as well as a fraction of apoB translocated into the ER undergo degradation. Therefore it is unlikely that secretion of apoB is regulated only through the translocation of the protein into the ER. Degradation of apoB could not be manipulated under any circumstances to make the cells to secrete 100% of synthesized apoB. The cells do not secrete more than about 80% of synthesized apoB under any condition, suggesting that degradation may occur at a basal level and provides another important regulatory point for apoB secretion (Yao and McLeod, 1994). By using a semipermeable system from HepG2 cells, the apoB degrading system was partially characterized in our laboratory (Adeli, 1994). Pulse-chase experiments were used to investigate posttranslational degradation of apoB. Results from these experiments showed that the apoB degrading system is highly pH and temperature sensitive, is stimulated by ATP, is located in a pre-Golgi compartment, and does not require calcium ion for its activity. Degradation of apoB could be effectively inhibited by the cysteine protease inhibitor, N-acetyl-leucyl-leucyl-norleucinal (ALLN). The sensitivity of the apoB degrading

system to ALLN has also been reported by a number of other investigators (Thrift *et al.*, 1992; Sakata *et al.*, 1993). Degradation of apoB in the ER of permeabilized HepG2 cells produced a number of fragments including a more abundant and stable fragment with a molecular weight of approximately 70 kDa. Subsequent experiments with different monoclonal antibodies raised against different epitopes on apoB showed that the 70 kDa fragment is an N-terminal portion of apoB (Sallach and Adeli, 1995).

1.8 Regulation of ApoB Production by Lipid Availability

Availability of lipids including triglycerides (Wu *et al.*, 1994a; Sato *et al.*, 1990; Boren *et al.*, 1993) and cholesteryl esters (Dashti, 1992; Cianflone *et al.*, 1990) have been pointed out as factors which can facilitate apoB translocation across the ER membrane and rescue it from degradation. Wu *et al.* (1994b) have concluded that cholesteryl ester does not play any role in the regulation of assembly and secretion of apoB from HepG2 cells. This contradicts the results obtained by Avramoglu *et al.* (1995) who have found a strong correlation between the intracellular mass of cholesteryl ester and apoB₁₀₀ secretion. They found a small relationship between the mass of intracellular TG and the rate of apoB secretion. Based on the available data from our lab and others on the assembly and secretion of apoB in HepG2 cells we have proposed a model for apoB biogenesis under lipid rich and lipid poor conditions (Adeli *et al.*, 1995). According to this model, lipids are added to apoB cotranslationally which helps proper folding and translocation of apoB across the ER membrane. The apoB assembled in the lipoprotein particles under lipid-rich conditions could be successfully secreted. However, an insufficient quantity of lipid results in the misfolding of the N-terminus of the apoB

molecule which subsequently impairs the apoB translocation into the ER. ApoB chains left in the ER membrane are then degraded by an ER- localized proteolytic system. This system also degrades apoB particles that have been released into the ER but are not secretion competent. Interestingly recent collaborative studies in our laboratory and Olofsson's group have shown that membrane bound and luminal apoB undergo different degradation pathways. The system degrading luminal apoB was sensitive to ALLN, whereas degradation of membrane bound apoB did not respond to ALLN and occurred at a faster rate compared to the luminal apoB (Adeli *et al.*, 1996).

1.9 Plan of Investigations

As it was discussed in the previous sections, production of apoB100 could be potentially regulated at several levels. Aside from transcriptional level which has already been ruled out in the previous investigations, apoB gene expression could potentially be regulated by modulation of apoB mRNA translation, translocation of apoB into the ER, or by degradation of apoB which has failed to properly assemble into lipoprotein particles. Our laboratory has been interested and involved in the investigation of regulation of apoB gene expression at different levels including, translation, translocation into the ER, and intracellular degradation of apoB. To investigate translational control of apoB production, *in vitro* synthesis of apoB in an mRNA dependent cell-free translation system is critical. Attempts towards translation of apoB mRNA in the reticulocyte lysate, i.e., the most common cell free system have not been successful (Olofsson *et al.*, 1985; Bostrom *et al.*, 1984; Reuben *et al.*, 1988). The reticulocyte lysate can not sustain the synthesis of proteins larger than 200 kDa (Pelham and Jackson, 1976). Since apoB100 is

synthesized exclusively in the liver, it is therefore reasonable to assume that an mRNA dependent system prepared from hepatocytes should be able to support the *in vitro* translation of apoB100 mRNA. The goal of the first part of this study was to prepare an mRNA dependent cell-free system from HepG2 cells which is capable of translation of apoB mRNA isolated from HepG2 cells.

Translocation of apoB across the ER membrane which is perhaps the most important regulatory point in apoB biogenesis, and degradation of apoB in a pre-Golgi compartment have also attracted much attention during the last few years. Availability of core lipids, triglycerides and cholesteryl esters has been implicated as a critical factor regulating apoB translocation across the ER membrane and its intracellular degradation. Since HMG-CoA reductase inhibitors deprive the cells from cholesterol, it seems therefore that these drugs should impair apoB transport into the ER and enhance its degradation. The second part of this study involved investigations on the effect of hypolipidemic drugs specially the new HMG-CoA reductase inhibitor, atorvastatin, on modulation of apoB secretion and degradation.

To study the intracellular degradation of apoB, a permeabilized system from HepG2 cells was developed in our laboratory which helped us to partially characterize the apoB degrading system in HepG2 cells (Adeli, 1994). Further studies on the characterization of apoB degrading system have been performed and are in progress in our laboratory. The third project includes part of the investigations on degradation of apoB bound to the ER membrane as well as degradation of different apoB subspecies

present in the ER lumen.

CHAPTER 2

Materials and Methods

2.1 Chemicals

Human hepatoblastoma cell line (HepG2 cells, ATCC HB 8065) were obtained from American Type Culture Collection (ATCC) (Rockville, M.D.) . Cell culture media. fetal bovine serum (certified grade) and immunoprecipitin were from Gibco BRL, (Life Technologies, Inc. Grand Island, N.Y.). Lysolecithin (L- α -Lysophosphatidylcholin. palmitoyl), albumin antiserum, creatine phosphokinase, phosphocreatine (di-Tris salt). dithiothreitol (DTT), spermidine trihydrochloride, L-amino acids (kit# LAA-21). adenosine 5'-triphosphate (dipotassium salt), guanosine 5'-triphosphate (sodium salt). ribonuclease A (type III-A), S-adenosyl-L-methionine, leupeptin, pepstatin A. benzamidine (hydrochloride), N-acetylleucylleucylnorleucinal (ALLN). soybean trypsin inhibitor, phenylmethyl sulphonyl flouride (PMSF), ammonium persulfate. ethylenediaminetetraacetic acid (disodium salt), deoxycholic acid (sodium salt). cytochrome c, β -nicotinamide adenine dinucleotide phosphate, reduced form (sodium salt), bromophenol blue (sodium salt), guanidium thiocyanate, sarcosyl, sodium citrate. sodium acetate, digitonin (50% purity) 2-mercaptoethanol, glycerol, high molecular weight standard mixture (cat.#SDS-6H), 5 β -cholan-24-oic acid-3 α ,12 α -diol (deoxycholic acid, sodium salt), fatty acid free albumin, oleic acid, ethidium bromide, pyridine-3-carboxylic acid (niacin; nicotinic acid), 2-[4-(2-[4-chlorobenzamido]ethyl)phenoxy]-2-methylpropanoic acid (Bezafibrate), 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic

acid 1-methyl ester (Fenofibrate), 2,2-dimethyl-5-[2,5-dimethylphenoxy]-pentanoic acid (Gemfibrozil), 2-(p-chlorophenoxy)-2-methylpropionic acid ethyl ester (Clofibrate). goat anti-human albumin antiserum (cat.#A-7544), goat anti-human α -1-antitrypsin (cat.#A-3897), rabbit anti-goat-IgG antiserum (cat.#G-5268), RNase inhibitor. agarose (molecular biology grade), and DEPC were from Sigma Chemical Company, (St. Louis, MO).

Ultra pure electrophoresis reagents; 40% acrylamide/bis solution. 37.5:1 (2.6% C) (cat.# 161-0148), N,N,N',N'-tetramethylethylenediamine (TEMED). tris (hydroxymethyl) aminomethane (Tris), sodiumdodecyl-sulfate (SDS), glycine, RNase-free sucrose and protein assay kit were from Bio-Rad Laboratories, (Richmond, CA).

L-[35 S]methionine (translation grade, specific activity of > 1100 Ci/mmol). was purchased from ICN, (Costa Mesa, CA).

EnhanceTM was obtained from Dupont Canada, (Toronto, ON).

Amplify was from Amersham International, (Amersham, UK).

Goat anti-human apoB serum (cat.#PBA0211) was from Medix Biotech Inc.. (San Carlos, CA).

Immunoprecipitin (10% v/v; formalin-fixed Steph A cells), Coomassie Brilliant Blue R-250, Eagle's minimum essential medium (alpha) powder, Earle's balanced salt solution powder, minimum essential medium without L-methionine/leucine/lysine, fetal bovine serum (certified grade), trypsin-EDTA (1X liquid), antibiotic-antimycotic (100X liquid), and sodium bicarbonate were obtained from Gibco BRL, (Life Technologies, Inc. Grand Island, N. Y.).

Ultra pure phenol for RNA extraction, ethylphenyl-polyethylene glycol (NP40, Nonidet P40), N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), and Triton X-100 were from United States Biochemical Corp (Cleveland OH).

Trichloroacetic acid, dimethyl sulphoxide, 2,5-diphenyloxazole (PPO), glacial acetic acid, isopropanol, methanol, ethanol, toluene, chloroform, isoamyl alcohol, standard buffer solutions (pH 4, 7, and 10), and other common laboratory reagents such as sodium chloride, sodium hydroxide, etc., were from British Drug House (BDH) Inc., (Toronto, ON).

Scintillation cocktail (Ready ProteinTM) was from Beckman, (Fullerton, CA). Proteinase K was purchased from Boehringer/Manheim Canada Ltd., (Laval, PQ). Trasylol (aprotinin) was from Bayer Leverkusen, FRG. Atorvastatin and lovastatin were provided by Dr. Roger S. Newton (Parke-Davis Pharmaceutical Research, Warner-Lambert Co., Ann Arbor, MI), oligo-dT cellulose spin column kit was purchased from 5 Prime → 3 Prime, Inc. (Boulder, CO). Dynabeads[®] Oligo (dT)₂₅ was from Dynal AS, Oslo. Brome Mosaic Virus RNA was from Promega (Madison, WI). Dialysis bag (MWCO: 6-8,000) was purchased from Spectra Medical Industries, Inc. (Houston, TX). Unless otherwise stated, all chemicals were of ACS grade.

2.2 Supplies

Disposable, sterile, tissue culture plates and dishes (6 well, 12 well, and 35, 60, 100 mm diameter), flasks (25 cm², 75 cm² and 175 cm²), disposable polypropylene centrifuge tubes (15 and 50 mL), disposable sterile serological pipettes (5 and 10 mL), were obtained from Corning (Corning, NY) or Fisher scientific (Pittsburgh, PA).

NalgeneTM sterile disposable syringe filters (0.2 and 0.4 μm) were from Nalgene Company, (Rochester, NY).

Vacu CapTM bottle-top filters (0.2 μm vacuum filters) were from Gelman Sciences Inc., (Ann Arbor, MI).

Kodak X-ray film exposure cassettes and X-OMAT AR films were obtained from Eastman Kodak Company, (Rochester, NY), Agfa CURIX XP films were from Agfa-Gevaert N.V. (Belgium).

Graduated polypropylene microcentrifugation tubes (1.5 or 2 mL) were purchased from Elkay Products Inc., (Shrewsbury MA), or United Scientific Products. (San Leandro, CA).

Micropipette tips were purchased from Canlab Scientific Products (Mississauga, ON).

Ultracentrifuge tubes (3, 5, and 13 mL) and disposable polyethylene scintillation counting vials were purchased from Beckman (Beckman Instruments Inc., Palo Alto, CA).

Cellophane sheets used in Bio-Rad GEL AIR gel drier were from Bio-Rad Laboratories, (Richmond, CA).

2.3 Equipment

Mass of chemicals was measured with an A&D electronic analytical balance with a sensitivity of ± 0.0001 g (Johns Scientific Inc., Toronto, ON) or an OHAUS portable electronic balance with a sensitivity of ± 0.01 g (Ohaus Corporation, Florham Park NJ).

A Corning pH meter (model 240) (Corning Science Products, Corning, NY) was used to measure and adjust the pH of solutions. The pH meter was calibrated before each and every measurement.

Cell culture was performed under a Nuaire class II, type A/B3 flowhood cabinet (Plymouth, MN), and a Nuaire Auto Flow CO₂ incubator was used to incubate the cells.

A Nikon TMS inverted phase-contrast microscope (Nikon Inc., Melville, NY) was used to observe the cells for counting, monitoring their growth, etc.

A hand tally counter (Taiwan) was used to count the cells.

Centrifuge machines used throughout the experiments include: Sorvall Ultra ProTM 80 (Dupont Co., Mississauga, ON), or the L8-55 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) for high speed centrifugations. Sorvall Super Speed RC2-B or a bench top clinical centrifuge for moderate and low speed centrifugation. Sorvall Microspin 24 S (Dupont Co., Mississauga, ON) for controlled speed microcentrifugation. Fischer Scientific (Ottawa, Ont) or Abbott TDX bench top microcentrifuges for fixed speed centrifugation.

A Beckman LS 7500 liquid scintillation counter, (Beckman Instruments Inc., Palo Alto, CA) was used for liquid scintillation counting.

Haake type W19 water baths, (Haake Circulators, Germany) were used to warm up tissue culture media, solutions used to treat HepG2 cells, and also for incubation of reactions in which temperatures more than room temperature were required.

Eppendorf adjustable micropipettes (Germany) were used for micropipetting of solutions. An eppendorf pipettor with eppendorf combitips (Brinkman Instruments Inc., Westbury, NY) was used for repetitive pipettings.

Electrophoresis of RNA samples was performed in a home made apparatus.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried using the SE250 Mighty SmallTM II Vertical Slab Unit, or the SE400 Sturdier Vertical Slab Unit purchased from Hoefer Scientific Instruments, (San Francisco, CA). The power supply systems used for electrophoresis were Power Pac 300 from Bio-Rad Laboratories, (Richmond, CA) the EC-103, or the EC-420 model from Mandel Scientific Co. Ltd., (Ville St. Pierre, PQ).

Electrophoresed gels were dried on a SE540 Slab Gel Dryer from Hoeffer Scientific Instruments (San Francisco, CA), or GEL AIR system from Bio-Rad Laboratories, (Richmond, CA).

A Gilford ResponseTM UV-VIS spectrophotometer (Ciba-Corning Diagnostics Corp., Oberlin, OH), and a 1.00 cm quartz cuvette (Hellma Canada., Concord, ON) were used for ultraviolet-visible absorbance readings and recordings.

Densitometric scanning of electrophoresis bands on X-ray films was performed with the Imaging Densitometer Model GS-670 by Bio-Rad Laboratories (Richmond, CA), and the results were analyzed by using the software accompanied by the instrument. loaded on an IBM clone computer.

2.4 Methods

2.4.1 Cell Culture

Reagents

Eagle's Minimum Essential Medium-alpha modification (α -MEM): The medium was purchased in a powder form (1X) and was prepared by adding distilled-deionized water and NaHCO_3 (2.2 g/L). The solution was stirred, the pH was adjusted to 7.1-7.2 (0.2-0.3 units below the desired final working pH), and sterilized by membrane filtration through Vacu CapTM bottle-top filters.

Earl's balanced salt solution (EBSS): EBSS was also obtained in a powder form (1X) and prepared as above.

Minimum Essential Medium minus methionine (MEM-meth): This medium was purchased in powder form as well (1X), deficient in methionine, leucine, and lysine. The medium was prepared by adding distilled-deionized water, L-leucine (52 mg/L), L-lysine (58 mg/L), and NaHCO_3 (2.2 g/L). After mixing the pH was adjusted (7.1-7.2), and filtered as above.

Fetal Bovine Serum (FBS): FBS was thawed at 37 °C, and heat inactivated at 56 °C for 30 minutes.

Complete Media: was prepared by addition of fetal bovine serum and antibiotics to α -MEM to a final concentration of 10% and 1%, respectively.

Procedure

Frozen HepG2 cells were thawed quickly at 37 °C and transferred to a 15-mL sterile centrifuge tube. Complete media (4 mL) was added dropwise while shaking the

tube gently. Cells were suspended 2X with a syringe (18_G1.5 gauge), and seeded into a T-75 flask containing 10 mL of complete medium. After mixing several times, cells were incubated in an atmosphere of 95% air, 5% CO₂. The medium was changed everyday for the first few days to remove any traces of dimethylsulfoxide (DMSO) (frozen cells were preserved in vials containing 25% serum, 65% α -MEM, and 10% DMSO to protect them against damages produced by freezing). Unless otherwise mentioned, the medium was changed every two to three days.

After reaching near confluence, cells were trypsinized by removing the medium to last traces, and then adding trypsin/EDTA (0.25% trypsin in 1mM EDTA) for 90 seconds. The trypsin was then removed completely and the flasks were incubated upright for 6 minutes at 37 °C in the incubator. The dislocated cells were then re-suspended in complete medium and homogenized by passing them through a 18_G1.5 gauge syringe for 6-8 times. This cell suspension was then diluted with complete media to the desired concentration of cells and used to subculture the cells into new flasks at a concentration of approximately 1×10^5 to 1.6×10^6 cells per flask depending on the size of the flask and the time that cells were needed for an experiment. The volume of complete medium added to the cells was: 2 mL/well for 6-well plates or 35 mm dishes, 4 mL for 25 cm² flasks, 12 mL for 75 cm² flasks. After 20-22 consecutive passages (i.e., 20-22 subcultures), cells were discarded and a new vial containing HepG2 cells was brought up from the -80 °C freezer.

After bringing up the cells from -80 °C freezer and thawing, the cells were subcultured at least two times before any experiment was performed.

2.4.2 Isolation of Total Cellular RNA

(Chomczynski and Sacchi method)

Reagents

Solution D (denaturing solution): 4M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (w/v) sarcosyl and 0.1 M 2-mercaptoethanol.

Phenol (water equilibrated): equilibration of water was performed according to Sambrook *et al.*, (1989). Phenol must be equilibrated to a pH > 7.8 since DNA will be removed by organic phase at this pH. Ultrapure phenol kept at -20 °C was thawed and then warmed to 68 °C in a water bath. Hydroxyquinoline, which is a partial inhibitor of RNase and a weak chelator of metal ions was then added to a final concentration of 0.1%. An equal volume of Tris-HCl buffer (0.5 M, pH 8.0) was then added and the solution was stirred for 15 min. on a magnetic stirrer. The solution was then left at room temperature until the two phases were separated. The upper aqueous phase was then aspirated. An equal volume of 0.1 M Tris-HCl, pH 8.0 was then added and the mixture was stirred for another 15 min.. The two phases were separated and the aqueous phase was aspirated again as above. The latter step was repeated until the pH of phenol solution became > 7.8. The phenol solution was then topped with 0.1 volume of 0.1 M Tris-HCl (pH 8.0) containing 0.2% b-mercaptoethanol and kept in a light-tight bottle at 4 °C.

Isopropanol (kept at -20 °C).

Ice-cold 75% ethanol.

Diethylpyrocarbonate (DEPC)-treated water, 0.1% (v/v).

Phosphate buffered saline (PBS).

Procedure

In this procedure total cellular RNA was isolated according to Chomczynski and Sacchi (1987). All reagents used for RNA extraction procedures were RNase free. Glassware and other materials used during the procedure were either baked in an oven at 180°C for at least 12-h, or were soaked in diethylpyrocarbonate (DEPC)-treated water for 2-h and then autoclaved or baked at 100 °C for 15 min. (Sambrook *et al.*, 1989).

Cultured cells were washed once with ice cold PBS, and then scraped in solution D (1 mL/25 cm² flask). Cells were then passed through a 20 gauge syringe 5X and the following were then added per mL of extract: 0.1 mL of 2 M sodium acetate, pH 4.0. 1 mL of water equilibrated phenol and 0.2 mL of chloroform-isoamyl alcohol (49:1 by volume). The suspension was vigorously mixed by shaking for 10 sec and cooled on ice for 15 min.. Protein and DNA were separated from RNA by centrifuging the extract at 10,000g for 20 min. at 4 °C. After centrifugation the aqueous phase containing the RNA was carefully transferred to a new tube. RNA was precipitated by addition of an equal volume of cold isopropanol and leaving the tube at -20°C for at least 1-h. The precipitated RNA was pelleted by centrifugation at 10,000g for 20 min. at 4 °C. For further purification, the RNA pellet was dissolved again in 300 µL of solution D, transferred to a microfuge tube, and re-precipitated as above. The RNA pellet was then resuspended in 75% ethanol, centrifuged, and vacuum dried for 15 min.. The purified RNA was dissolved in 500 µL of DEPC-treated water and the purity of RNA preparation was checked by measuring the A_{260/280} nm ratio. A ratio of approximately 2 indicates a

relatively pure RNA preparation. The RNase activity in the sample was also examined as described below.

2.4.3 Isolation of Total Cellular RNA

(Han *et al.* method)

Reagents

5M guanidinium thiocyanate stock: The solution was prepared by mixing 60 g of guanidinium thiocyanate with 5 mL of 1M Tris-HCl; pH 7.5, 25 mL of 0.1 M EDTA, and 8 mL of 2-mercaptoethanol (added just before use), in a total volume of 100 mL.

6M guanidine hydrochloride: prepared by mixing 57 g of the salt with 25 mL of 0.1 M EDTA, pH 7.0, and 75 μ L of 2-mercaptoethanol in a total volume of 100 mL.

Procedure

This procedure was performed according to Han *et al.* (1987) with slight modifications. Three T-75 flasks of cultured HepG2 cells were used to extract RNA. Cells were washed once with ice cold PBS and scraped in ice cold 5M guanidinium thiocyanate (2 mL/flask). Cell suspensions were combined in a graduated, precooled, 50 mL polypropylene centrifuge tube and immediately homogenized with ice-cooled polytron for 20 s at maximum speed. The tube was covered with parafilm to prevent the sample from splashing. The contents were then poured in a 13-mL Corex tube containing 1.8 mL of cold ethanol (-20°C), mixed and immediately centrifuged in a HB4 rotor (precooled to -10°C) at 10,000 rpm for 5 min.. The supernatant solution was mixed with 150 μ L 1M acetic acid and 4.5 mL cold ethanol (-20°C) and incubated at -20°C for at least 5h. The precipitated RNA was then pelleted by centrifugation at 6000 rpm for 10

min. at -10°C . The supernatant was discarded and the pellet was resuspended in 1 mL of cold (-20°C) 6M guanidinium hydrochloride (Gdn-HCl) by repeated pipetting. The volume was adjusted to 2 mL and 50 μL of 1 M acetic acid and 1 mL of cold (-20°C) ethanol were added. The contents were mixed and incubated at -20°C for more than 3h and centrifuged as above. This step was repeated two more times with decreasing the volume of guanidinium hydrochloride to 1 mL and volumes of 1 M acetic acid and ethanol to 25 μL and 500 μL , respectively. A small aliquot (about 30 μL) of Gdn-HCl solution containing RNA was used to test for RNase activity as described below. After this test the RNA pellet was dissolved in 500 μL of DEPC treated water. A small aliquot (~ 10 μL) was used to measure the $A_{260/280}$ nm ratio and the rest was kept at -80°C until use. The RNase activity in the sample was then examined as described below.

2.4.4 Isolation of Cytoplasmic RNA

Reagents

RNA extraction buffer: 0.14 M NaCl, 1.5 mM MgCl_2 , 10 mM Tris-HCl (pH 8.6), 0.5% Nonidet P-40 (NP-40), 1 mM dithiothreitol, 1000 units/mL placental RNase inhibitor or 20 mM vanadylribonucleoside complexes.

Protein digestion buffer: 0.2 M Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.3 M NaCl, 2% SDS.

Proteinase K stock: 20 mg/mL in water, kept at -20°C .

Phosphate buffered saline (PBS).

This procedure was performed according to Sambrook *et al.*, (1989). All steps were performed on ice unless mentioned otherwise. Two T-75 culture flasks containing

HepG2 cells with about 80% confluence were used for RNA extraction. After removing the medium, HepG2 cells were washed twice with 5-7 mL of ice-cold PBS lacking calcium and magnesium ions. Cells were then scraped into the residual PBS left in the flasks and transferred to a microfuge tube. PBS was then added to bring the volume to 1 mL, the tube was centrifuged at 12,000g for 30 s at 4 °C. The supernatant was discarded and the cell pellet was resuspended in 200 µL of RNA extraction buffer, vortexed for 15 s and left on ice for 5 min.. The suspension was then centrifuged at 12,000g for 90 s at 4 °C. The supernatant was transferred to a fresh microfuge tube and after addition of 200 µL of proteinase digestion buffer and proteinase k (to a final concentration of 50 µg/mL) the solution was mixed and incubated at 37 °C for 30 min.. The proteins were then removed by extracting the solution once with an equal volume of phenol:chloroform. and centrifuging the tube at 5000g for 10 min. at room temperature. The aqueous supernatant was transferred to a fresh microfuge tube, 400 µL of ice-cold isopropanol were added and after mixing the tube was chilled on ice for 30 min.. RNA was then pelleted by centrifugation at 12,000g for 10 min. at 4 °C. The supernatant was carefully remove with a micropipettor and RNA pellet was resuspended in 1 mL of 70% ethanol by briefly vortexing at room temperature and pelleted again by recentrifugation. The supernatant was removed completely and the open tube was kept at room temperature in a desiccator until the last visible traces of ethanol evaporated. The RNA was then dissolved in 100 µL of distilled, deionized, DEPC treated water, an aliquot was used to measure the RNA concentration and the $A_{260/280}$ nm ratio and the rest was kept at -80 °C until use. The RNase activity in the sample was examined as described below.

2.4.5 Examination of RNA Samples for RNase Activity

Reagents

50 mM sodium acetate, pH 7.0 prepared in DEPC treated water.

TBE buffer: 89 mM Tris, 89 mM boric acid, 10 mM EDTA.

Procedure

This procedure was performed according to Han *et al.* (1987). The aliquot containing RNA (about 30 μ L) was precipitated with ethanol, centrifuged and the pellet was dissolved in the same volume of DEPC treated sodium acetate, and incubated at 37 $^{\circ}$ C for 30 min.. The RNA sample was then loaded on a 1.5% nondenaturing agarose gel (in TBE buffer, containing 0.5 μ g ethidium bromide/mL), and electrophoresed at 80 V. for 1-h. The presence of 28S and 18S rRNA bands in a ratio of approximately 2:1 indicates the presence of intact RNA and lack of RNase activity. If the ratio is less than 1:1, further cycles of purification procedure should be performed to remove the residual RNase activity.

2.4.6 Preparation of PolyA⁺ RNA Using Oligo(dT) Cellulose Spin Columns

Reagents

The columns and reagents used in this procedure were purchased as a kit. The kit contained Oligo(dT) cellulose spin columns, collection tubes, collection tube stoppers and the following reagents:

Loading buffer: 2.5 M NaCl, 100 mM Tris-HCl, 5 mM EDTA, pH 7.5.

0.5 M NaCl buffer: 0.5 M NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.5.

0.1 M NaCl buffer: 0.1 M NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.5.

Elution buffer: 10 mM Tris-Cl, 1 mM EDTA, pH 7.5.

Storage buffer: 20 mM Tris-Cl, 1 mM EDTA, 0.02% Thimerosal, pH 7.5.

Molecular biology grade RNase-free water.

Washing solution: 0.1 M NaOH, 5 mM EDTA.

3M Sodium acetate, pH 5.2, sterile, RNase-free.

Mussel glycogen: 1 mg/mL in distilled H₂O.

Procedure

This procedure to isolate poly A⁺ mRNA was performed according to the manufacturer instructions.

Preparing the column

All reagents and columns were allowed to reach the room temperature before using them.

The resin was fully suspended by inverting the column several times. The top and the bottom closures were removed and the column was placed in a RNase-free collection tube and spun in a bench top centrifuge at about 200g for 10 s at room temperature. The top and bottom closures were saved as they were used again during the procedure. The buffer accumulated in the collection tube was discarded and the column was returned to the collection tube. The column was recapped and 2 mL of 0.5 M NaCl buffer was added to the resin and the above procedure was repeated again.

Preparing the RNA sample

RNA sample, up to a maximum amount of 1 mg (1 A₂₆₀ unit = 40 µg of RNA) was dissolved in 2 mL of molecular biology grade water (supplied with kit), and was

heated at 65 °C for 5 min.. Loading buffer (0.25 volume) was then added to the RNA sample and mixed. The RNA sample was kept on ice until it was applied to the Oligo (dT)-cellulose column.

Running the Column

Elution buffer (3.5 mL) was poured in an RNase-free tube, capped, and kept at 65 °C until use. The RNA sample was applied on the pre-spun column, mixed thoroughly with the Oligo(dT) cellulose by repeated inversions, and incubated at room temperature for 10 min. with mixing every 2-3 min. intervals. After this period, the caps were removed, the column was placed in the collection tube and centrifuged as described. The solution in the collection tube which contains the poly A⁻ was discarded. The column was washed 3 times, each time by adding 2 mL of 0.5 M NaCl buffer, suspending the resin and spinning as above. The column was washed two more times, each time with 2.0 mL of 0.1 M NaCl buffer as described. The Oligo(dT) cellulose column was then transferred to a new RNase-free collection tube and poly A⁺ RNA was eluted from the column by applying 1 mL of 65 °C elution buffer and centrifuging at 200g for 10 s. The elution of RNA was repeated one more time and in the same collection tube. According to the manufacturer, 90% of the poly A⁺ mRNA will be eluted at this step. The remainder of poly A⁺ mRNA was eluted in a new collection tube by washing the column once with pre-warmed elution buffer as described above.

Ethanol precipitation of poly A⁺ mRNA

To each collection tube, 20 µL of 1mg/mL mussel glycogen, 0.1 volume of 3 M sodium acetate; pH 5.2, and 2.5 volume of cold (-20 °C) 95-100% ethanol was added and

the tube was incubated at -20°C for at least 2-h. The RNA was then pelleted by centrifugation at 16,000g for 30 min. at 0°C . The pellet was washed twice with cold 70% ethanol and once with 95-100% ethanol and then dried at room temperature until last visible residues of ethanol were evaporated. The poly A⁺ RNA was then dissolved in DEPC treated water a small aliquot was used to measure the RNA concentration and the rest was kept at -80°C until use.

2.4.7 Preparation of PolyA⁺ RNA Using Dynabeads[®] Oligo (dT)₂₅

Dynabeads[®] Oligo (dT)₂₅ are uniform beads made of polystyrene with a diameter of about 2.8 μm . The beads are superparamagnetic and a chain containing 25 deoxythymidylate is covalently attached to them via a 5'-linker group. The beads bind approximately 2 mg polyA⁺ RNA per mg. The beads are supplied as a suspension in phosphate-buffered saline (PBS) containing 0.02% NaN_3 .

Reagents

Phosphate buffered saline (PBS), pH 7.4 (solution A): 137 mM NaCl, 2.7 mM KCl, 4.3 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.4 mM KH_2PO_4 .

Lysis buffer (solution B): 10 mM Tris-HCl (pH 7.5), 0.14 M NaCl, 5 mM KCl, 1% Triton X-100 or NP-40.

Lysis/Binding buffer (solution C): 100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA; pH 8.0, 1% LiDS (SDS), 5 mM dithiothreitol (DTT).

2X binding buffer (solution D): 20 mM Tris-HCl (pH 7.5), 1.0 M LiCl, 2 mM EDTA, 0.4-1.0% SDS.

Washing buffer (solution E): 10 mM Tris-HCl (pH 7.5), 0.15 M LiCl, 1 mM EDTA, 0.1-0.3% SDS.

Elution buffer (solution F): 2 mM EDTA (pH 7.5).

Procedure

In this method the cells are lysed gently with a mild detergent such as NP-40 or Triton X-100, and nuclei are removed by centrifugation. mRNA is then recovered directly from the nuclei-free, cytoplasmic supernatant.

After removing the medium, cells were washed twice with ice cold PBS. The PBS was removed by aspiration and cells were scraped in residual PBS with a teflon cell scraper, and transferred to a microcentrifuge and kept on ice. Cells were pelleted by centrifuging the tube and 2000g for 5 min.. Lysis buffer (100 μ L) was added to the pellet. vortexed for 10-15 seconds and the tube was placed on ice for 1 min.. After this the tube was centrifuged at 12000g for 30 seconds and the supernatant was transferred to a RNase-free microfuge tube containing about 350-450 μ g Dynabeads[®] Oligo (dT)₂₅ in 100 μ L of 2X binding buffer. The mRNA was allowed to bind to the beads for 5 min., the tube was then placed in the Dynal MPC[®]-E and the supernatant was removed. Beads were washed 2-3 times with 0.2 mL of washing buffer using the Dynal MPC[®]-E. The last washing buffer was removed completely. Elution buffer (100 μ L) was added to the beads and the tube was incubated at 65 ⁰C for 2 min.. The tube was then placed in Dynal MPC[®]-E and the supernatant containing the mRNA was transferred to a new RNase-free microfuge tube. The mRNA was then precipitated by adding an equal volume of cold (-20 ⁰C) isopropanol and chilling the tube on ice for 30 min.. The mRNA was pelleted by

centrifugation at 12000g for 10 min. at 4 °C. The supernatant was completely removed and RNA was resuspended in 100 µL of 70% ethanol and recentrifuged. The supernatant was removed carefully and the open tube was left at room temperature (in a desiccator) to until the last visible residues of ethanol were evaporated. mRNA was then dissolved in 50-100 µL of DEPC treated, distilled, deionized water. A small aliquot (about 5 µL) was used to measure RNA concentration and the rest was kept at -80 °C until use.

2.4.8 *In vitro* Translation of Endogenous mRNA

Reagents

Buffer A: 150 mM RNase-free sucrose, 33 mM NH₄Cl, 7mM KCl, 1.5 mM Mg (OAc)₂, 30 mM Hepes, pH 7.4.

Lysolecithin: 150 mg/mL prepared fresh in buffer A.

Translation buffer: 100 mM Hepes; pH 7.4, 200 mM KCl, 7 mM NH₄Cl, 0.5 mM Mg(OAc)₂, 1 mM dethiotreitol, 1 mM ATP, 1 mM GTP, 40 µM of each of 19 amino acids minus methionine, 0.1 mM S-adenosylmethionine, 1 mM spermidine trihydrochloride, 10 mM creatine phosphate, 40 units/mL of creatine phosphokinase. The solution was aliquoted and kept in -80 °C.

RNase A Solution: 3 mg/mL stock solution; stored at -20 °C.

Protease Inhibitor Cocktail: 1 mM benzamidine, 5 mM EDTA, 0.86 mM PMSF, 100 Kallikrein inactivating units/mL of aprotinin, 10 mM Hepes, 50 µg/mL leupeptin, 50 µg/mL pepstatin. The stock cocktail mix was stored in aliquotes at -80 °C.

Procedure

Monolayer HepG2 cells about 75-80% confluent in 25-cm² (or 75-cm²) flasks were depleted of methionine by incubating them in MEM minus methionine for 60 min. at 37 °C under 5% CO₂. Preparation of cell-free lysate was based on the method of Brown *et al.*, (1983) with some modifications. To prepare the lysate, cells were washed twice with 5 mL (for a 75 cm² flask) of ice-cold buffer A, and 10 mL of lysolecithin solution was then added to the flask, and incubated on ice in a flat and level position for 75 s. The lysolecithin solution was aspirated and the flask was left on an angle for 30 s on ice, and the residues of lysolecithin was also aspirated. The lysed cells were scraped in 750 µL (250 µL for 25-cm² flask) of translation buffer and transferred to a RNase-free fresh microcentrifuge tube. After repeated pipetting (~ 15X) to suspend the cells, the lysate was centrifuged at 12000g for 1 min. at 4 °C. The supernatant was transferred to a RNase-free fresh tube and immediately used for translation reaction.

In vitro protein synthesis in cell-free extracts of HepG2 cells was carried out in the presence of 400 µCi/mL of [³⁵S]methionine, at 30 °C for 60 min.. A control was always included in the experiment by adding RNase A (final concentration 20 µg/mL) to a small aliquot of the lysate. Immediately after translation, the lysate was mixed with a protease inhibitor cocktail (8 µL/100 µL of lysate). Radioactive incorporation was determined by trichloroacetic acid (TCA) precipitation as described below.

2.4.9 Preparation of mRNA-Dependent HepG2 Cell-Free System

Reagents

Buffer A: 150 mM RNase-free sucrose, 33 mM NH_4Cl , 7mM KCl, 1.5 mM Mg (OAc)₂, 30 mM Hepes, pH 7.4.

Lysolecithin: 150 mg/mL prepared fresh in buffer A.

Hepes buffer: 0.1 M, pH 7.4.

Procedure

To prepare the mRNA dependent translation system, monolayer HepG2 cells about 75-80% confluent were used. The cytoplasmic membranes of HepG2 cells were lysed by lysolecithin as described above, after removal of the lysolecithin from the flask. the lysed cells were suspended by scraping them in 0.4 mL of Hepes buffer. After repeated pipetting (15X), the lysate was centrifuged at 4 °C and 12000g for 1 min.. The supernatant was collected and used immediately for translation or preparation of nuclease treated (mRNA-dependent) lysate. Endogenous mRNA was hydrolyzed by incubating the lysate with micrococcal nuclease at a ratio of 4 units of enzyme per 1 A_{260} unit of polysomes, in the presence of 1 mM Ca^{2+} for 7-10 min., at 20 °C. Typically the lysate had approximately 25 units of polysomes per mL. One unit absorbency at 260 nm was considered to be equivalent to one unit of polysomes (Giannakouros and Georgatsos, 1988). The enzyme was inhibited by the addition of EGTA to a final concentration of 2 mM. The nuclease-treated lysate could be kept at -80 °C. Lysates used after three months from preparation were as active as the fresh lysate towards the translation of the

exogenous mRNA. Nuclease-treated lysates kept for more than three months were not tested.

2.4.10 *In vitro* Translation of Exogenous mRNA

Reagents

Translation buffer: 100 mM Hepes; pH 7.4, 200 mM KCl, 7 mM NH₄Cl. 0.5 mM Mg(OAc)₂, 1 mM dethiotreitol, 1 mM ATP, 1 mM GTP, 40 µM of each of 19 amino acids minus methionine, 0.1 mM S-adenosylmethionine, 1 mM spermidine trihydrochloride, 10 mM creatine phosphate, 40 units/mL of creatine phosphokinase. The solution was aliquoted and kept in -80 °C.

Protease Inhibitor Cocktail: 1 mM benzamidine, 5 mM EDTA, 0.86 mM PMSF. 100 Kallikrein inactivating units/mL of aprotinin. 10 mM Hepes, 50 µg/mL leupeptin. 50 µg/mL pepstatin. The stock cocktail mix was stored in aliquotes at -80 °C.

Procedure

Protein synthesis in the mRNA dependent HepG2 lysates was carried out in the presence of 400 mCi of [³⁵S]methionine/mL of lysate, at 30 °C for 1.5-2-h. mRNA dependent lysate was mixed with translation buffer at a ratio of (2:3) and then supplemented either with poly A⁺ RNA (12-24 mg/mL final concentration) or cytoplasmic total RNA (200 mg/mL final concentration). KCl (100 mM) and Mg (OAc)₂ (4.5 M) were also added to the mixture so that the final concentration of K⁺ and Mg²⁺ became 150 and 1 mM, respectively. Immediately after the incubation period, lysate was mixed with protease inhibitor cocktail 80 µL/mL to prevent proteolysis in the lysate. Radioactive incorporation into the newly synthesized proteins was determined by TCA

precipitation. Intact lysate (lysate not treated with micrococcal nuclease) containing 0.5 mg/mL of RNase A and mRNA dependent lysate without exogenous RNA were also used as controls.

2.4.11 Immunoprecipitation of Proteins from Translation Products

Reagents

Solubilization buffer: PBS containing 1% NP40, 1% deoxycholate, 5mM EDTA, 1 mM EGTA, 2 mM PMSF, 0.1 mM leupeptin, 2 mg/mL ALLN.

Immunoprecipitation was carried out as previously described (Adeli, 1994). *In vitro* translation products (100-500 μ L) were mixed with nine volumes of solubilization buffer and were immunoprecipitated by addition of 10 μ L of polyclonal antibody (anti-apoB, or albumin, diluted with PBS at a ratio of 1:5). The specificity of these antibodies has already been extensively characterized in our laboratory by immunoblotting analysis of human plasma and HepG2 cell extracts and shown to have no cross-reactivity with other apolipoproteins. Immunoprecipitation was allowed to proceed overnight at 4 $^{\circ}$ C. After this incubation, 2 μ l of rabbit anti-goat IgG was added and the mixture was further incubated for 1-h at 4 $^{\circ}$ C. Immunoprecipitin (30 μ l) was added and the incubation continued for 1-h at room temperature. The immunoprecipitin was separated by centrifugation for 1 min. in a microcentrifuge and the pellet was washed four times with immunoprecipitation wash buffer (PBS containing 1% NP-40, 1% deoxycholate, 5mM EDTA, 0.1% SDS). Finally, the immunoprecipitates were prepared for SDS-PAGE by suspending and boiling the pellet in 60 μ l of electrophoresis sample buffer (Laemmli, 1970).

2.4.12 SDS-PAGE and Fluorography

Reagents

Destaining solution: 50% distilled water, 40% methanol, 10% acetic acid.

Discontinuous SDS-PAGE was performed essentially as described (Laemmli, 1970). The slab gels were composed of 5% (w/v) stacking (3% when apoB was to be identified) and 6 or 10% (w/v) resolving gels. After electrophoresis the proteins were fixed in the gels by incubating the gel in destaining solution for at least 30 min. and fluorographed after incubating in the Enhance (Dupont). The gels were dried using a vacuum gel dryer or the Bio-Rad AirGel dryer, and exposed to Kodak X-Omat AR5 film at -80 °C for 1-4 days. Molecular weight markers (Sigma) were carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase b (97.4 kDa), β -galactosidase (116 kDa), myosin (205 kDa), and purified LDL-apoB (550 kDa). An alternative molecular weight marker (rainbow marker, Bio-Rad) also was used in the experiments which contained: myosin (208 kDa), β -galactosidase (144 kDa), bovine serum albumin (87 kDa), carbonic anhydrase (44.1 kDa), soybean trypsin inhibitor (32.7 kDa), lysozyme (17.7 kDa), and aprotinin (7.1 kDa) [The apparent molecular weight of the proteins is larger than the pure unmodified proteins, because they represent the molecular weight of protein-dye complexes].

2.4.13 Treatment of HepG2 Cells with Hypolipidemic Drugs

Lipid-lowering drugs, *atorvastatin*, and *lovastatin* were prepared as a 10 mM solution in ethanol, and were diluted to 1 mM with sterilized, distilled, deionized water. *Clofibrate*, *bezafibrate*, *gemfibrozil*, and *probucol* were dissolved in 50% ethanol to a final

concentration of 400 µg/mL, 120 µg/mL, 240 µg/mL, and 200 µg/mL, respectively. *Nicotinic* acid was dissolved in sterile, distilled, deionized water and neutralized with NaOH, the volume was then adjusted to give a final concentration of 12 mg/mL. To treat the cells with drugs, medium was changed and drug solutions were added to the fresh medium to obtain the desired concentration.

2.4. 14 Metabolic Labeling and Permeabilization of HepG2 Cells

Reagents

Digitonin: 50 µg/mL prepared by dissolving the powder in 50% ethanol with warming the solution.

CSK buffer: (0.3 M sucrose, 0.1 M KCl, 2.5 mM MgCl₂, 1 mM Na-free EDTA, 10 mM PIPES, pH 6.8). To prepare the CSK buffer, the PIPES buffer was made first, and after pH adjustment to 6.8, other ingredients were added to the PIPES buffer.

[³⁵S]methionine protein-labeling mix.

Procedure

Preparation of permeabilized HepG2 cells was performed according to Adeli (1994). Near confluent HepG2 cultures grown in 100 mm dishes were washed two times with EBSS or methionine free MEM and then incubated with methionine-free MEM for 60 min. at 37 °C under 5% CO₂. HepG2 cells were pulse-chased and made semipermeable as described below. Cells were incubated with methionine free MEM containing 50-150 µCi/mL of [³⁵S]methionine for the appropriate period of time. The [³⁵S]methionine-containing medium was then aspirated and cells were, washed in Earl's

balanced salt-solution three times, and chased in complete medium containing 5-10 mM methionine for 10 min. to complete the nascent apoB chains. The cells were then washed with and incubated in CSK buffer containing 50 $\mu\text{g/mL}$ of digitonin for 10 min. at room temperature. Digitonized cells were washed three times in CSK buffer and were used for the degradation studies.

The concentration of digitonin, the temperature, and the time required for effective permeabilization of cell membranes without damaging the ER membrane during the permeabilization process have already been optimized in our laboratory (Adeli, 1994). Measurement of the activities of lactate dehydrogenase (cytosolic marker) and NADPH-cytochrome c reductase (ER marker) in the CSK buffer showed that treatment of HepG2 cells with digitonin at 50 $\mu\text{g/mL}$ results in the maximal loss of cytosolic components without damage to the ER. Under these conditions the activity of the lactate dehydrogenase was maximal, while only negligible amount of the NADPH-cytochrome c reductase activity was detected in the CSK (Adeli, 1994).

2.4.15 Monitoring ApoB Degradation in Permeabilized HepG2 Cells

After permeabilizing with digitonin, HepG2 cells were used to observe apoB degradation under different conditions. Depending on the experiment, the cells were incubated in CSK buffer for various periods of time. At the end of incubation period, cells were prepared for immunoprecipitation of apoB or other proteins.

2.4.16 Immunoprecipitation of Proteins for Secretion and Degradation

Studies

Reagents

Solubilization buffer: Each mL of the solubilization buffer contains: 450 μ L of 5XC buffer (250 mM Tris-HCl, pH 7.4, 750 mM NaCl, 25 mM EDTA, 5mM PMSF, 5% Triton-X100), 512 μ L of PBS, 25 μ L of Trasylol, and 12.5 μ L of PMSF.

Wash buffer: 10 mM Tris-HCl, pH 7.4, 2mM EDTA, 0.1% SDS, 1% Triton X-100.

Procedure

At the end of incubation period, cells were scraped in 500 μ L of solubilization buffer and homogenized by passing them 6 times through a syringe with a 21G needle. The lysate was then centrifuged at 12000g and 4 $^{\circ}$ C for 1 min. and the supernatant was transferred to a fresh tube. Cell lysates or fractions collected from sucrose gradients were then diluted with 800 μ L of the solubilization buffer and were immunoprecipitated as follows. Preimmune serum (2 μ L) was added to the lysate and incubated at room temperature for 2-h. After the incubation period, 60 μ L of Immunoprecipitin was added and the lysate was incubated for an additional hour. Samples were then cleared by centrifuging in a microfuge for 2 min., and the supernatant was transferred to a new tube for immunoprecipitation of apoB or other proteins. Immunoprecipitation was performed by first adding 10 μ L of antibody or antiserum (diluted 5X with PBS) to each sample and incubating overnight at 4 $^{\circ}$ C. Immunoprecipitin (100 μ L) was then added to each sample and further incubated at room temperature for 1-h. Samples were centrifuged for 2 min. at

14000 rpm to pellet the immunoprecipitates. The pellet was washed three times with the wash buffer by resuspending in the wash buffer and centrifuging as above. The immunoprecipitates were prepared for SDS-PAGE by suspending and boiling in 100 μ l of electrophoresis sample buffer (Laemmli, 1970).

2.4.17 Isolation of LDL from Human Plasma

Reagents

Heavy density salt solution: contained 153g NaCl, 354g KBr. and 0.1g EDTA per liter (density: 1.346)

0.15 M sodium chloride solution (density: 1.005 g/mL)

Salt solutions (d: 1.019, and d: 1.063 g/mL): These solutions were prepared by dilution of heavy density solution with 0.15 M NaCl according to the formula below.

Procedure

LDL was isolated from human plasma according to the method of Havel *et al.* (1955). The density of plasma (1.006 g/mL) was adjusted to 1.019 g/mL by adding heavy density solution to the plasma according to following formula:

$$V1.d1 + V2.d2 = (V1 + V2)d$$

where V1 and d1 are the volume and density of plasma, V2 and d2 are the volume and density of heavy density solution and d is the desired density (1.019 g/mL). The plasma was then transferred to ultracentrifuge tubes. The tubes were filled with salt solutions of equal density and centrifuged for 24-h at 4 °C at 49,000 rpm in the TH-641 rotor of the Sorval Ultra Pro 80 centrifuge. After centrifugation the top layer containing VLDL and chylomicrons was carefully removed by aspiration and the plasma was transferred to

fresh ultracentrifuge tubes. The density was adjusted to 1.063 g/mL, the tubes were filled with salt solution of equal density and centrifuged at 40,000 rpm for 24-h at 4 °C. The top layer containing LDL was removed very carefully and prepared for dialysis.

Dialysis of LDL was performed using dialysis bags with 6.4 mm diameter. The bags were first soaked in boiling water for 10 min. and then washed extensively with distilled, deionized water. LDL samples were then loaded and dialyzed against 4L of PBS for 24-h, changing the PBS after 12-h. LDL samples were then aliquoted in sterilized microfuge tubes and nitrogen gas was blown gently through the sample. The tubes were then capped and sealed with parafilm and were kept in the refrigerator until they were used for experiment.

2.4.18 Preparation of Albumin-Oleate Complex

To treat the HepG2 cell cultures with oleate, a complex of albumin-oleate was prepared according to the method of Van Harken *et al.* (1969). Fatty acid free bovine serum albumin (BSA) (150 mg) was dissolved in 10 mL of methionine deficient medium containing 5% FBS. After leaving for five minutes, albumin was dissolved by gentle mixing of solution. The medium was then sterilized by filtering through a 0.45 µm syringe filter. The medium containing albumin was then added to 40 mL of methionine deficient medium + 5% FBS in a sterile 50 mL tube.

In a microfuge tube, 34 mg of oleic acid was dissolved in 500 µl of ethanol. 75 µl of the oleic acid solution was then added to the BSA containing medium. The medium was incubated overnight at room temperature, or at 37 °C before use. This medium was used within a day.

The BSA containing medium without oleic acid was used to treat the control cells.

2.4.19 Separation of Luminal and Membrane Fractions of Microsomes

Subcellular fractionation was performed as previously described (Boren *et al.*, 1990; Boren *et al.*, 1992; Bostrom *et al.*, 1988; Bostrom *et al.*, 1986). All solutions were chilled on ice and all steps were performed on ice unless mentioned otherwise. Permeabilized cells incubated for 0-2-h were washed once with 250 mM sucrose, 3 mM imidazole, pH 7.4 and once with 50 mM sucrose, 3 mM imidazole, pH 7.4. The cells were then scraped in 0.5 mL of 50 mM sucrose solution supplemented with a cocktail of protease inhibitors (0.1 mM leupeptin, 1 mM PMSF, 100 KIU/mL Trasylol, 1 μ M pepstatin A. and 5 μ M ALLN) and homogenized in a glass Dounce homogenizer with 15 strokes. Sucrose (50 μ l, 49%) was added and cells were further homogenized by additional 5 strokes. The homogenate was transferred to microfuge tubes and centrifuged for 10 min. at 2200g. The supernatant containing the crude microsomes was transferred to a new microfuge tube and the pellet was suspended in 250 μ l of 250 mM sucrose and centrifuged as above. The supernatant was added to the supernatant from first centrifugation and the volume was adjusted to 1.55 mL with 250 mM sucrose. This solution containing microsomes was then subjected to sodium carbonate extraction to release the contents of microsomes. To which 1 mL of PBS, 150 μ l of 49% sucrose, and 300 μ l of 1 M sodium carbonate were added, and after proper mixing, the solution was left at room temperature for 25 min.. Fatty acid free albumin (25 mg) was then added to the tube and after waiting for 5 min., the solution was mixed properly. The tubes were then balanced and centrifuged in the TH-641 rotor of Sorval Ultra Pro 80 at 35,000 rpm

and 21 °C for 93 min.. After centrifugation, the supernatant was decanted in a clean tube and the centrifuge tube was tapped on a paper towel against the bench top to remove all the residues of the supernatant. Acetic acid (180 µl, 10% v/v) was then added to neutralize the luminal fraction. The luminal fraction was then used either for immunoprecipitation of total apoB or it was subjected to sucrose-gradient ultracentrifugation to separate different apoB100-containing particles.

2.4.20 Sucrose Gradient Ultracentrifugation of ApoB100-Containing Lipoproteins

In some experiments luminal contents isolated from microsomes were supplemented with protease inhibitors (0.1 mM leupeptin, 1 mM phenylmethylsulfonyl flouride, 100 KIU/mL Trasylol, 1 µM pepstatin A, and 5 µM ALLN) and then subjected to ultracentrifugation on a step sucrose gradient. Beckman ultracentrifuge tubes (12.5 mL) were used for this purpose and they were filled from the bottom to the top with: 1.5 mL of 49% sucrose, 3 mL of 25% sucrose, 2 mL of 20% sucrose, 3.5 mL of sample, 1.9 mL of 5% sucrose and 0.9 mL of imidazole buffer. The tubes were then balanced in an analytical balance by using the imidazole buffer and were centrifuged for 65h in the TH-641 rotor of Sorval Ultra Pro 80 at 35,000 rpm and 12 °C for 65h. After centrifugation, 1-mL fractions were collected from the bottom of the tubes using a home made fraction collector. These fractions were then used to immunoprecipitate apoB100 or other proteins.

2.4.21 Immunoprecipitation of ApoB from Microsomal Membranes

Solubilization buffer: each mL of the solubilization buffer contains: 450 µl of 5XC buffer (250 mM Tris-HCl, pH 7.4, 750 mM NaCl, 25 mM EDTA, 5mM PMSF. 5% Triton-X100), 512 µl of PBS, 25 µl of Trasylol, and 12.5 µl of PMSF.

The membrane pellet was first suspended in 1 mL of PBS by repeated pipetting. 800 µl of solubilization buffer was then added and the suspension was further homogenized by using a syringe with 20G needle. The homogenate was then centrifuged for 10 min. in a microcentrifuge at speed and the supernatant was transferred to a fresh tube for protein immunoprecipitation.

2.4.22 Quantitative Measurement of Radioactivity in Protein Bands in SDS-PAGE Gels.

This procedure was performed essentially according to Wettsten *et al.* (1985). The dried gels were taped to the X-ray films and were kept in -80 °C for appropriate exposure period. Before detaching the gels from X-ray films for development, a few triangle cuts were made on at least two sides of the gel and film. The film was then separated from gel and developed. After drying the X-ray film, the film was stapled (at the bottom side) on the back of the gel matching the cuts on the gel, with those of the film. A carbon copy paper, facing the back of the gel was then inserted between the film and the gel, then a white page of paper was inserted between the film and carbon paper to make the protein bands visible. A transparent plastic sheet was then overlaid on top of the film and using a pen, a box was made around the chosen bands. This way the location of the bands were transferred on the back of the gel.

Using a pair of scissors, these bands were then cut out of the gel and transferred into the scintillation vials. To each vial, 400 μ l of 30% hydrogen peroxide and 200 μ l of perchloric acid was then added and the vials were incubated overnight at 60 $^{\circ}$ C. After this incubation, 5 mL of scintillation cocktail were added to vials and after vortexing, radioactivity was counted using a Beckman LS 7500 liquid scintillation counter.

CHAPTER 3

RESULTS

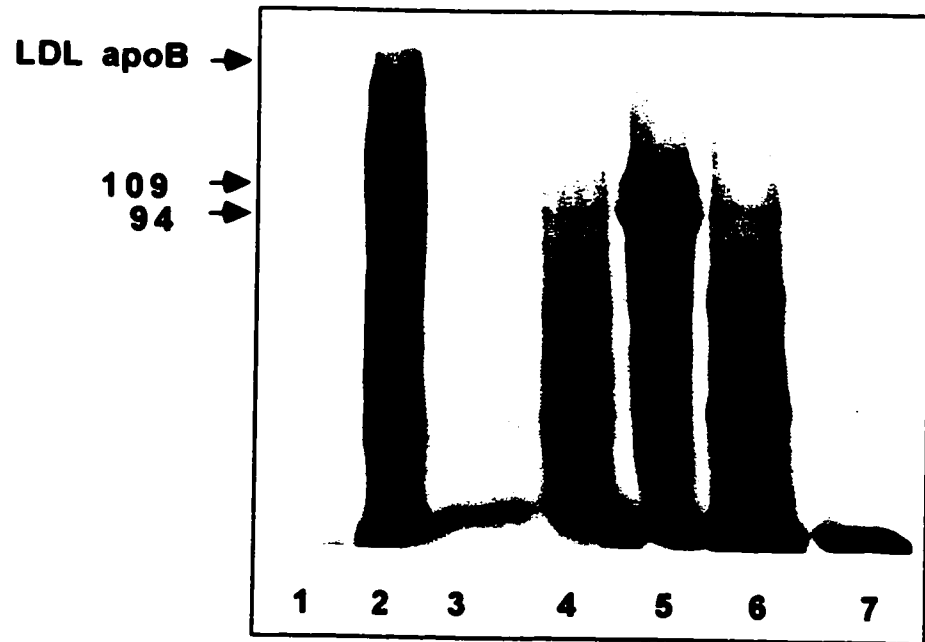
3.1 *In vitro* Translation of ApoB mRNA in a mRNA-Dependent HepG2 Cell-free System

3.1.1 Preparation and Characterization of a mRNA Dependent HepG2 Lysate

Cell-free lysate from HepG2 cells was prepared as described in Chapter 2 (under preparation of mRNA-dependent HepG2 cell-free system). A fraction of the lysate was used for translation of endogenous mRNA and another fraction was used for micrococcal nuclease treatment. The translational activity of mRNA-depleted HepG2 lysate was examined by the addition of Brome Mosaic Virus (BMV) RNA as control to the lysate. BMV RNA is commonly used as a positive control to examine the translational efficiency of cell-free lysates (Ahlquist *et al.*, 1984). The viral RNA was added to the translation reaction mixture at a concentration of 10 µg/mL. The major BMV proteins, i.e., the 109 and 94 kDa proteins (Ahlquist *et al.*, 1984) were synthesized and clearly visible on the fluorograph. Figure 3.1 shows the fluorograph of the SDS-PAGE of *in vitro* synthesized proteins in the untreated HepG2 cell-free lysate and in the nuclease treated HepG2 lysate after addition of different RNA samples. No polypeptides were synthesized when translation was carried out in the untreated lysate in the presence of 200 µg/mL of RNase A (control number 1, lane 1). The untreated lysate incorporated radiolabeled methionine into polypeptides ranging in size from 20 to 550 kDa (apoB100, lane 2). The micrococcal nuclease-treated lysate without added RNA did not synthesize any proteins,

Figure 3.1**Translation of Exogenous mRNA in Nuclease Treated Lysate.****Legend**

HepG2 lysate was prepared and treated with micrococcal nuclease (4U/1U of polysomes) as described in Materials and Methods. Translation was carried out in the presence of 400 μ Ci [35 S]methionine/mL of intact or micrococcal nuclease treated lysate. Translation products were then analyzed by SDS-PAGE (5% stacking and 6% resolving gels) and fluorography. Lane 1 represents translational products of HepG2 cell lysate treated with RNase A; lane 2, translation products in fresh intact lysate (untreated lysate); lane 3, translation products in lysate treated with micrococcal nuclease; lane 4, translation products in nuclease treated lysate supplemented with polyA⁺ RNA prepared by Dynal magnetic beads; lane 5, translation products in nuclease treated lysate supplemented with BMV RNA; lane 6, translation products in nuclease treated lysate supplemented with polyA⁺ RNA prepared by Oligo (dT) spin columns; lane 7, translation products in nuclease treated lysate supplemented with total cellular RNA.

Figure 3.1

showing complete digestion of endogenous mRNA by micrococcal nuclease (control number 2, lane 3). BMV mRNA was actively translated in the nuclease treated lysate as evidenced by high level of radioactivity incorporation (11 fold stimulation over control. Table 3.1) and the pattern of translation products of BMV RNA (Figure 3.1, lane 5).

Addition of poly A⁺ RNA prepared by Dynal magnetic beads and oligo (dT) spin columns (lanes 4 and 6, respectively) induced the synthesis of radiolabeled polypeptides up to a molecular weight of approximately 90 kDa. However, larger proteins were not synthesized, perhaps due to the loss of the RNA during purification of poly A⁺ RNA from total RNA or due to the physical damage to apoB mRNA during this procedure. The lysate did not respond to the total cellular RNA (Chomczynski and Sacchi, 1987) and no polypeptides were synthesized (lane 7). When the lysate was supplemented with total cytoplasmic RNA (Sambrook *et al.*, 1989), a band matching the apoB from human LDL indicating the synthesis of full length apoB appeared on the fluorograph (Figure 3.2, lane 2).

Sometimes a band corresponding to a protein matching the 94 kDa protein of BMV also appeared on all lanes (Figure 3.1). Since this protein was also present in RNase treated lysates, it is hypothesized that the 94 kDa band is the product of a nontranslational mechanism resulting in the binding of labeled methionine to an unlabeled protein in the lysate. Quantitative incorporation of labeled methionine in newly synthesized proteins in intact and nuclease treated lysates under above conditions is represented in Table 3.1.

Figure 3.2**Synthesis of ApoB in Nuclease-Treated Lysate****Legend**

HepG2 lysate was prepared and treated with micrococcal nuclease (4U/1U of polysomes) as described in Materials and Methods. The lysate was supplemented with cytoplasmic RNA or BMV RNA and translation was carried out in the presence of 400 μ Ci [35 S]methionine/mL of lysate. Translation products were then analyzed by SDS-PAGE (3% stacking and 6% resolving gels) and fluorography. Lane 1 represents translation products obtained when the lysate was incubated in the presence of RNase A. Lane 2 shows the translation products obtained in nuclease treated lysate supplemented with cytoplasmic RNA. Lane 3 shows translation products of lysate supplemented with BMV RNA.

Figure 3.2

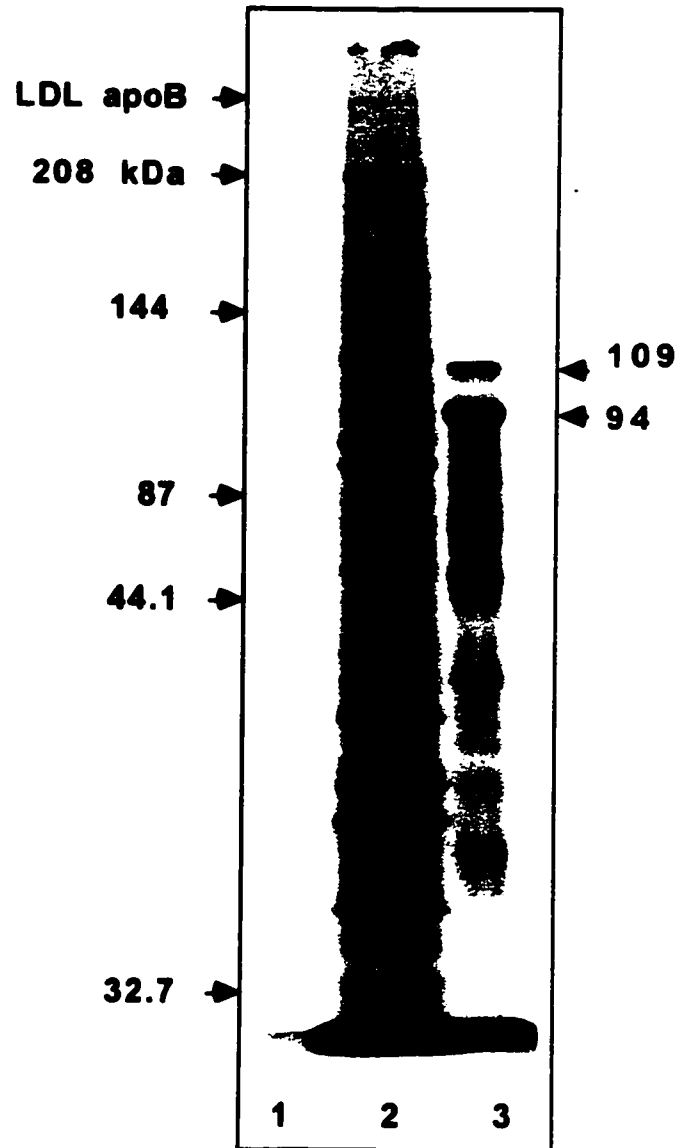


Table 3.1**Incorporation of Labeled Methionine in the Newly Synthesized Proteins Under
Different Conditions****Legend**

HepG2 lysate was prepared and treated with micrococcal nuclease (4U/1U of polysomes) as described in Materials and Methods. Translation was carried out in the presence of 400 μCi [^{35}S]methionine/mL of intact or micrococcal nuclease treated lysate. 5 μl of lysate was then used for TCA precipitation and radioactivity count as described in Materials and Methods.

Table 3.1

Sample	cpm/ml
Control	5.35×10^3
Intact lysate	3.53×10^6
Nuclease treated lysate	1.03×10^6
Lysate supplemented with poly A ⁺ RNA (beads)	2.3×10^6
Lysate supplemented with poly A ⁺ RNA (spin columns)	1.48×10^6
Lysate supplemented with total RNA	1.2×10^6
Lysate supplemented with BMV RNA	5.96×10^6

3.1.2 Optimization of the Translation Reaction in Nuclease-Treated HepG2

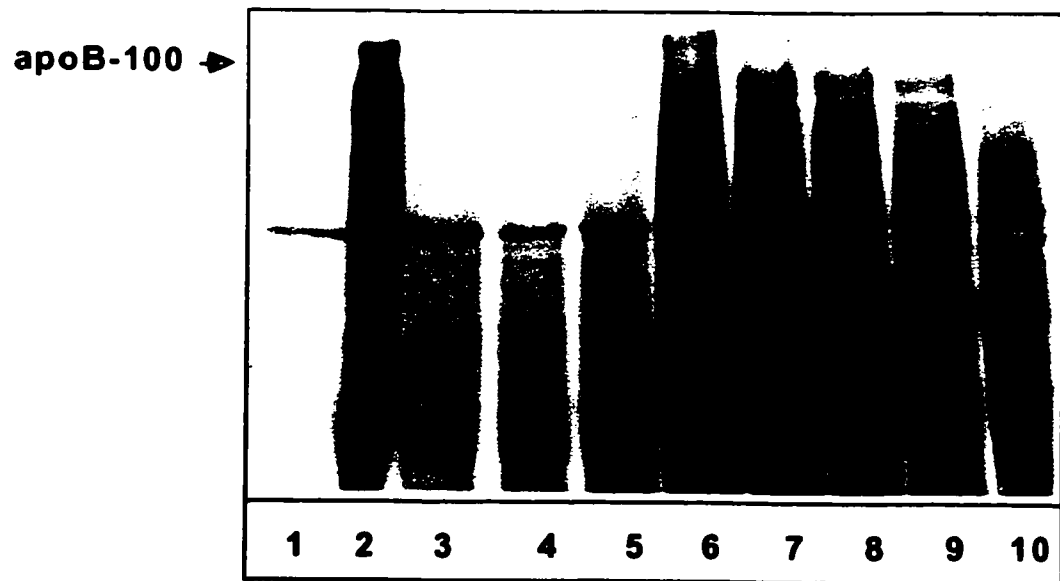
Lysate

Since protein synthesis in cell free systems is very sensitive to the ionic environment (Jagues, 1987; Jackson and Hunt, 1983), the optimum concentrations of K^+ and Mg^{2+} required for the translation of mRNAs of high molecular weight proteins such as apoB100 in the mRNA-dependent lysate were also investigated. The optimum concentration of K^+ and Mg^{2+} for translation of endogenous mRNA in HepG2 lysate were found to be 200 and 1 mM, respectively (Theriault, 1994). Different concentrations of Mg^{2+} between 0.3-3 mM were examined. In the presence of 1-2.5 mM Mg^{2+} the lysate was able to accommodate the synthesis of proteins as large as apoB100, with the maximum activity being at 1 mM Mg^{2+} (Fig 3.3, lanes 6-9). Concentrations higher than 2.5 mM of Mg^{2+} inhibited translation of large proteins such as apoB₁₀₀ (Fig 3.3. lane 10). The system However, was not very sensitive to moderate changes in the K^+ concentration. Changing the K^+ concentration from 150 mM to 200 mM, resulted only in a slight change in the radioactivity counts from 3.9×10^6 cpm/mL of lysate to 4.3×10^6 cpm/mL of lysate in the presence of 200 μ g of cytoplasmic RNA per mL of lysate.

Typically [35 S]methionine at a concentration of 400 μ Ci/mL of lysate was used in all experiments. Increasing concentration of [35 S]methionine to 800 μ Ci/mL of lysate resulted in an increase up to 80% in the translational activity of the mRNA-dependent lysate as evidenced by the increase in the radioactivity counts (8.25×10^6 versus 4.6×10^6 cpm/mL of lysate). Increasing the time of incubation of the lysate at 30 $^{\circ}$ C from 1-h to 1.5h

Figure 3.3**Effect of Mg^{2+} Concentration on the Translational Activity of HepG2 mRNA-Dependent Translation System****Legend**

Treatment of the lysate with micrococcal nuclease and translation were carried out as described in Materials and Methods. Lane 1, lysate treated with RNase A. Lane 2, intact lysate. Lane 3, lysate treated with micrococcal nuclease. Lanes 4-10, micrococcal nuclease treated lysates supplemented with cytoplasmic HepG2 RNA (200 μ g/mL) and a final concentration of Mg^{2+} of 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mM, respectively.

Figure 3.3

also resulted in a higher incorporation of [^{35}S]methionine into the TCA precipitable polypeptides (9.3×10^6 versus 6.3×10^6 cpm/mL of lysate).

3.1.3 Nuclease-Treated Lysate Sustains the Synthesis of Full-length Polypeptides

As indicated in Figure 3.4, the nuclease-treated lysate supplemented with cytoplasmic RNA extracted from HepG2 cells was found to synthesize full-length albumin and apoB100 as evidenced by immunoprecipitation with polyclonal antibodies against albumin and apoB. Lane 1 and 2 represent apoB immunoprecipitated from intact lysate (not treated with micrococcal nuclease) and mRNA-dependent lysate supplemented with cytoplasmic RNA, respectively. The size of apoB immunoprecipitated from mRNA-dependent lysate is identical to the apoB immunoprecipitated from intact lysate. Translation products of mRNA-dependent lysate supplemented with poly A⁺ RNA isolated from total cellular RNA by Oligo dT spin columns is shown in lane 3. Lane 4 shows albumin immunoprecipitated from translation products of poly A⁺ RNA. The poly A⁺ RNA preparation was not able to sustain the synthesis of apoB as mentioned previously.

3.1.4 Protease Activity in the HepG2 Cell-Free Lysate

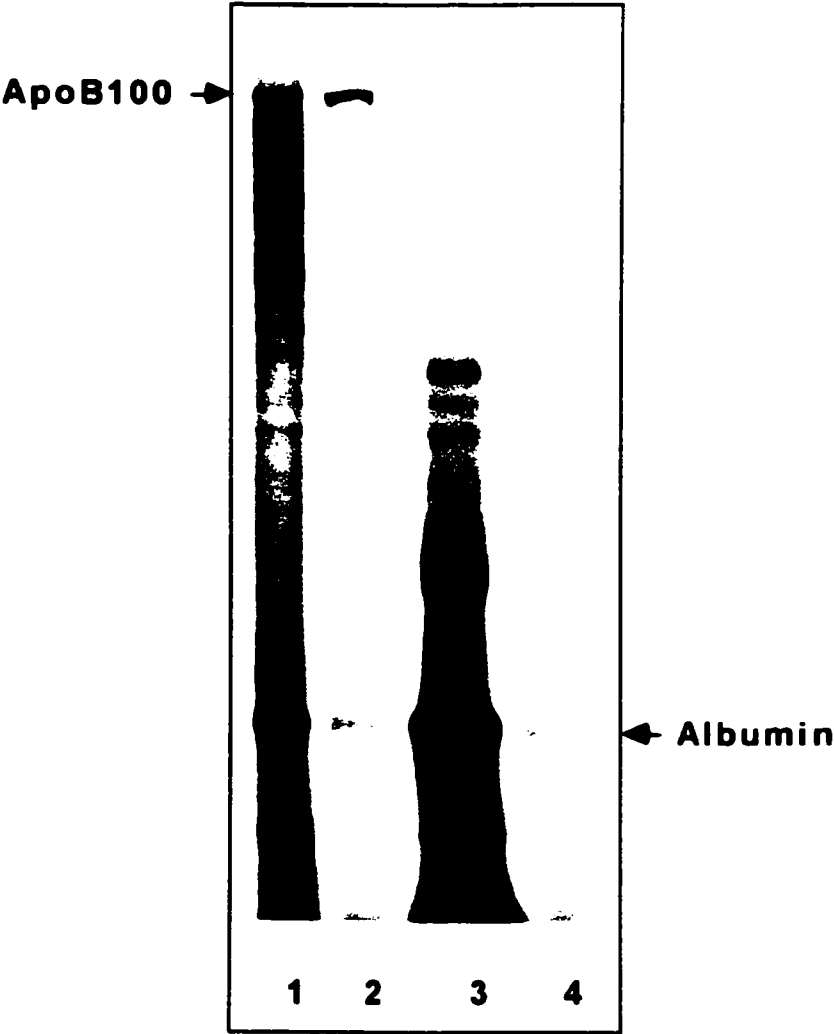
The HepG2 cell-free system previously developed in our laboratory was extensively studied and characterized. The addition of a battery of protease inhibitors, including ALLN, leupeptin, pepstatin, and soybean trypsin inhibitor did not influence the translational activity of the lysate or the pattern of translation products, indicating no protease activity in the HepG2 lysate (Adeli and Theriault, 1992; Theriault, 1994). In the

Figure 3.4**Translation Products of Cytoplasmic and Poly A⁺ HepG2 RNA and Immunoprecipitation of ApoB100 and Albumin****Legend**

HepG2 lysate was prepared and treated with micrococcal nuclease (4U/1U of polysomes) as described in Materials and Methods. Translation was carried out in the presence of 400 μCi [³⁵S]methionine/mL of intact or micrococcal nuclease treated lysate.

After translation reaction, lysates were mixed with solubilization buffer as follows: intact lysate (100 μl + 900 μl of solubilization buffer); nuclease treated lysate supplemented with cytoplasmic RNA (1mL + 1mL of solubilization buffer); nuclease treated lysate supplemented with polyA⁺ RNA (100 μl + 900 μl of solubilization buffer). ApoB100 or albumin were immunoprecipitated overnight using 10 μl of a polyclonal antibody (diluted 1:5 with PBS). Electrophoresis was performed according to LaemmLi (1970). Gels were dried after electrophoresis and exposed to Kodak X-Omat AR5 film. Lane 1, apoB100 immunoprecipitated from translation products of intact lysate. lane 2, apoB100 immunoprecipitated from translation products of mRNA-dependent HepG2 lysate supplemented with cytoplasmic RNA. Lane 3, translation products of mRNA-dependent lysate supplemented with poly A⁺ RNA, lane 4, albumin immunoprecipitated from translation products of lysate supplemented with poly A⁺ RNA.

Figure 3.4



present study, the effect of two other protease inhibitors, aprotinin and phenylmethylsulfonylfluoride (PMSF) were also investigated. No difference was observed in the pattern of translation products in the presence or absence of protease inhibitors (Figure 3.5). The effect of protease inhibitors was also assessed quantitatively. An aliquot of the protease inhibitor-treated lysates and untreated lysate were counted after TCA precipitation of proteins. The results showed no significant change in protein synthesis activity in the presence or absence of protease inhibitors (Table 3.2).

3.1.5 Translocation of ApoB into the Microsomes in the HepG2 Lysate

The presence of microsomal membranes and the ability of the untreated lysate to transport the newly synthesized proteins into the microsomes has already been investigated in our laboratory. By measuring the activity of the ER-specific enzyme, NADPH-cytochrome c reductase, it has been shown that the cell free system contains a considerable amount of microsomes. Based on two experiments, enzyme activity was shown to be between 35.0 to 40.7 nmol/min/mg of lysate protein. Separation of microsomal membranes and cytosolic fraction by ultracentrifugation showed that the majority of the enzyme is bound to the microsomal membranes (88.8 vs 9.1 nmol/min/mg of lysate protein). The integrity of the microsomal membranes and the ability of the membranes to translocate *in vitro* synthesized proteins was also investigated by using a protease protection assay. Protease protection, and immunoprecipitation assays showed that a number of small and medium size proteins, including apoAI, apoE, and albumin were translocated into the microsomes following their translation (Theriault, 1994). In the present study the ability of the system to translocate the apoB into the microsomes

Figure 3.5**Protease Activity in the HepG2 Cell-free Lysate****Legend**

Cell-free lysate was prepared from near confluent HepG2 cells. Translation of endogenous RNA was performed as described in Materials and Methods in the absence (lane 1) and presence of aprotinin (lane 2), and phenylmethanesulfonylfluoride (PMSF) (lane 3).

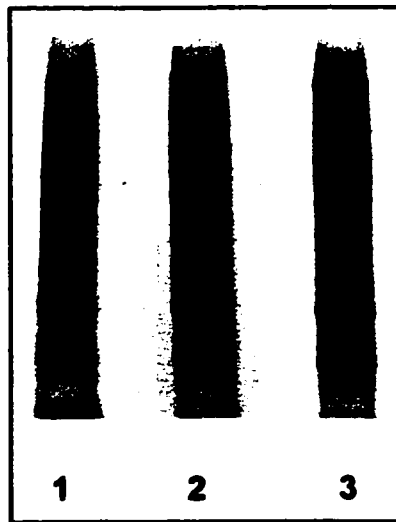
Figure 3.5

Table 3.2
Protease Activity in HepG2 Lysate

Legend

HepG2 lysate was prepared from intact HepG2 cells as described in Materials and Methods. Translation was carried out in the presence of 400 μCi [^{35}S]methionine/mL of lysate in the presence of 5 $\mu\text{g/mL}$ aprotinin or 1 mM PMSF. After the incubation, 5 μl of lysate was used for TCA precipitation and radioactivity counting as described in Materials and Methods.

Table 3.2

Sample	cpm/ml
control lysate	5.2×10^7
lysate + aprotinin	5.4×10^7
lysate + PMSF	5.2×10^7

was investigated. Protease protection assays revealed that about 40% of apoB was also transported into the microsomes present in the intact lysate.

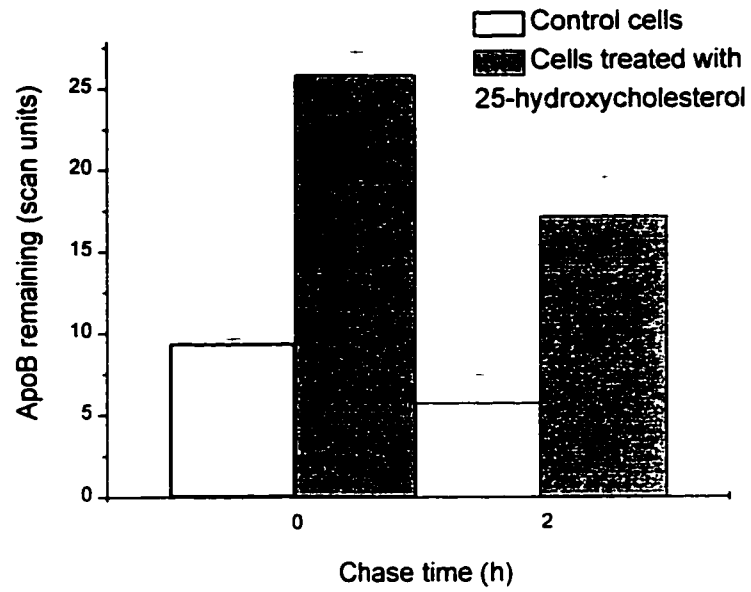
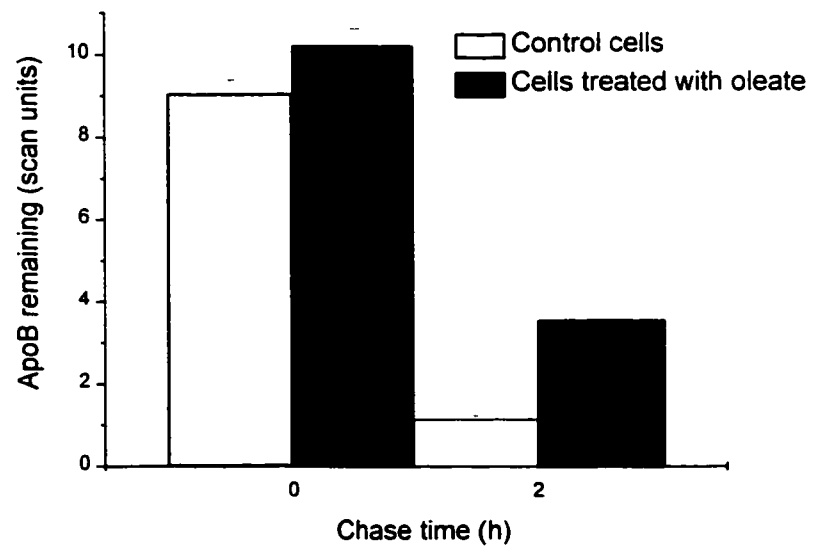
3.2 Effect of 25-Hydroxycholesterol and Oleate on ApoB Turnover in HepG2

Cells

Effect of atorvastatin on apoB degradation was examined under normal and lipid rich conditions. Since oleate (Dixon *et al.*, 1991; White *et al.*, 1992; Furukawa *et al.*, 1992) and 25-hydroxycholesterol (Dashti, 1992) have already been shown to increase apoB secretion by different cell lines, oleic acid and 25-hydroxycholesterol were used as sources of lipid. Monolayer HepG2 cells were treated with lipids overnight and degradation experiments were performed the next day. Figure 3.6A represents the effect of 25-hydroxycholesterol on apoB degradation. Although after 2-h of chase the percent apoB degraded in 25-hydroxycholesterol treated cells was comparable to control cells, at 0 time (after the first chase in α -MEM) the amount of apoB synthesized in 25-hydroxycholesterol treated cells was three times that of the control cells, suggesting stimulation of apoB synthesis. In oleic acid treated cells, (panel B) the amount of apoB synthesized in treated cells at 0 time was almost equal to the control cells. However, after 2-h of chase, the amount of apoB remaining in treated cells was more than three times that of control cells. This indicates protection of apoB from degradation by oleic acid. It was therefore decided to use oleic acid as the source of lipid to treat HepG2 cells.

Figure 3.6**Effect of 25-Hydroxycholesterol and Oleate on ApoB Turnover in HepG2 Cells****Legend**

Monolayer HepG2 cells were treated with A) 25-hydroxycholesterol or B) oleic acid (0.3 mM) overnight. Cells were pulsed, chased, permeabilized and incubated in CSK as described in Materials and Methods. After harvesting, cells were homogenized in solubilization buffer and apoB was immunoprecipitated from supernatant after spinning the homogenate at 4 °C for 1min. at 12000g. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer and results were plotted as apoB remaining at each time period.

Figure 3.6**A****B**

3.3 Modulation of ApoB Secretion and Degradation by Hypolipidemic Drugs

3.3.1 Effect of Nicotinic acid, Probucol, and Fibric Acid Derivatives

The first group of drugs investigated for their effects on apoB degradation were: nicotinic acid, clofibrate, bezafibrate, probucol, and gemfibrozil. Drug solutions were prepared as described in Materials and Methods, and HepG2 cells were treated for 4 days. The final concentrations of drugs in the cell culture medium were: 600, 300, 120, 200, and 120 $\mu\text{g/mL}$, respectively. Treatment started when cells were about 50-60% confluent. Cells treated with nicotinic acid, bezafibrate and probucol were almost confluent at the time of harvest. However, gemfibrozil treated cells showed very little growth.

In the first step, apoB degradation was studied in intact cells. To prevent protein secretion, HepG2 cells were treated with 1 $\mu\text{g/mL}$ of brefeldin A throughout the pulse-chase and incubation of the cells thereafter. Figure (3.7) shows the amount of apoB remaining in the cells after 2-h of chase. Compared to control, clofibrate, gemfibrozil, nicotinic acid, and probucol showed: 13.9, 16.8, 26.6, and 29.5% decrease in the amount of apoB remaining, respectively. However, bezafibrate showed a 22.2% increase in apoB remaining over the control. Percent apoB remaining in control, and drug treated cells are shown in Table 3.3. Analysis of data with a *t*-test did not show any significant difference between control and drug treated cells ($p < 0.05$).

To further investigate the effect of hypolipidemic drugs on apoB degradation, a semipermeable system developed from HepG2 cells in our laboratory (Adeli, 1994) was employed. Treatment conditions and concentration of drugs were the same as those for

Figure 3.7**Effect of Hypolipidemic Drugs on ApoB Degradation in Intact HepG2 Cells****Legend**

HepG2 cells were treated with nicotinic acid (600 $\mu\text{g/mL}$), clofibrate (300 $\mu\text{g/mL}$), bezafibrate (120 $\mu\text{g/mL}$), probucol (200 $\mu\text{g/mL}$), and gemfibrozil (120 $\mu\text{g/mL}$). Treatment was started when the cells were about 50% confluent and continued for 4 days. Cells were then pulsed, chased, and incubated in complete medium as described in Materials and Methods. Brefeldin A at a concentration of 1 $\mu\text{g/mL}$ was present throughout pulse, chase, and 2-h incubation to prevent protein secretion by the cells. Cells were then harvested and homogenized in solubilization buffer; after centrifugation apoB was immunoprecipitated from the supernatant as described in Materials and Methods. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer and results were plotted as percent apoB remaining after 2-h in each condition compared to the same condition at time 0 (before 2-h incubation). Cont.: control, B: bezafibrate, Clo.: clofibrate, G: gemfibrozil, N: nicotinic acid, P: probucol.

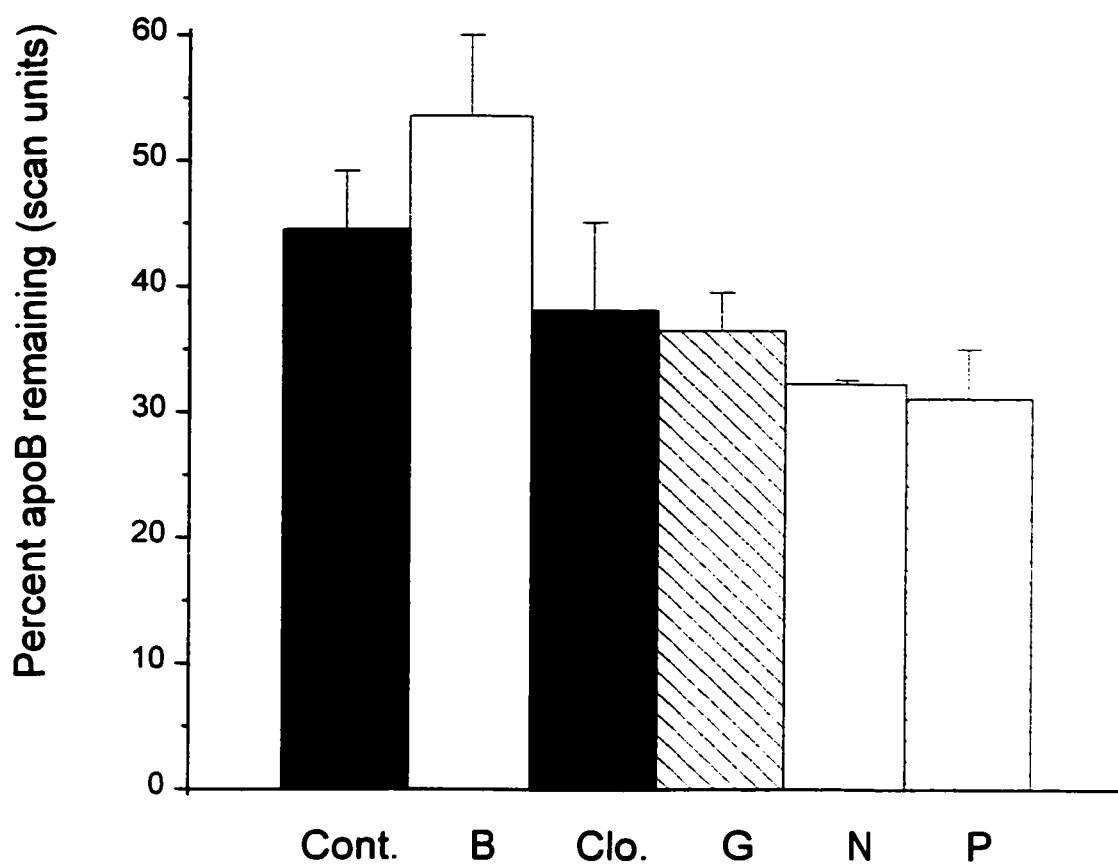
Figure 3.7

Table 3.3

**Percent ApoB Remaining in Intact HepG2 Cells Treated with Nicotinic Acid,
Probucol, and Fibrate Derivatives**

Legend

HepG2 cells were treated with nicotinic acid (600 µg/mL), clofibrate (300 µg/mL), bezafibrate (120 µg/mL), probucol (200 µg/mL), and gemfibrozil (120 µg/mL). Treatment was started when the cells were about 50% confluent and continued for 4 days. Cells were then pulsed, chased, and incubated in complete medium as described in Materials and Methods. Brefeldin A at a concentration of 1 µg/mL was present throughout pulse, chase, and 2-h incubation to prevent protein secretion by the cells. Cells were then harvested and homogenized in solubilization buffer and after centrifugation apoB was immunoprecipitated from supernatant as described in Materials and Methods. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer. percent apoB remaining after 2-h in each condition was calculate based on the amount of apoB present in the same condition at time 0 (before 2-h incubation). Analysis of data with a *t*-test did not show any significant difference between control and drug treated cells ($p < 0.05$). The results are expressed as: mean \pm SD.

Table 3.3

	Control cells	Bezafibrate	Clofibrate	Gemfirozil	Nicotinic acid	Probucol
% apoB remaining *	44.56±4.60	53.6±6.4	38.15±6.95	36.53±3	32.3±0.22	31.15±3.9

* Analysis of data by Student's t-test ($p < 0.05$) showed no significant difference between control and drug treated cells.

intact cells. Pulse, chase and permeabilization of the cells were performed as described in Materials and Methods. Figure (3.8) represents the results of the effect of hypolipidemic drugs on apoB degradation in permeabilized HepG2 cells. The average results of 4 experiments showed that bezafibrate, clofibrate, gemfibrozil, and nicotinic acid decreased the percent apoB remaining by 8.2, 57, 57.9, and 10.7%, respectively, compared to control cells. However, cells treated with probucol showed an increase of 8.5% in percent apoB remaining compared to control cells. Table 3.4 represents percent apoB remaining in control and drug treated cells. Analysis of data with Student's t-test and analysis of variance showed that only cells treated with gemfibrozil and clofibrate had significantly different degradation rates compared to control cells.

Based on the results obtained from the above experiments, two drugs, gemfibrozil and clofibrate, were chosen for further investigations. In experiments with gemfibrozil, HepG2 cells were treated with 120 $\mu\text{g/mL}$ of gemfibrozil for 4 days. Pulse, chase and immunoprecipitation of apoB were performed as described in Materials and Methods. Figure (3.9) shows the results of gemfibrozil treatment on apoB degradation in permeabilized HepG2 cells. There was a 43.8% decrease in apoB immunoprecipitated at 0h in gemfibrozil treated cells compared to control cells. Albumin was also immunoprecipitated to examine whether this decrease was specific to apoB or it was the result of a general decrease in protein synthesis in HepG2 cells. Figure (3.10) represents the amount of immunoprecipitated albumin at each time period. A substantial decrease was observed in the amount of albumin present in the drug-treated cells compared to control cells at each time period. It was therefore concluded that gemfibrozil at the

Figure 3.8**Effect of Hypolipidemic Drugs on ApoB Degradation in Permeabilized HepG2 Cells****Legend**

HepG2 cells were treated with nicotinic acid (600 $\mu\text{g/mL}$), clofibrate (300 $\mu\text{g/mL}$), bezafibrate (120 $\mu\text{g/mL}$), probucol (200 $\mu\text{g/mL}$), and gemfibrozil (120 $\mu\text{g/mL}$). Treatment was started when the cells were about 50% confluent and continued for 4 days. Cells were then pulsed, chased, and permeabilized with 50 $\mu\text{g/mL}$ digitonin as described in Materials and Methods and were incubated in CSK buffer at 37 $^{\circ}\text{C}$ for 2-h. Cells were then harvested and homogenized in solubilization buffer and after centrifugation for 1 min. at 4 $^{\circ}\text{C}$ and 12000g, apoB was immunoprecipitated from supernatant as described in Materials and Methods. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer and results were plotted as percent apoB remaining after 2-h in each condition compared to the same condition at time 0 (before 2-h incubation). Cont.: control, B: bezafibrate, Clo.: clofibrate, G: gemfibrozil, N: nicotinic acid, P: probucol.

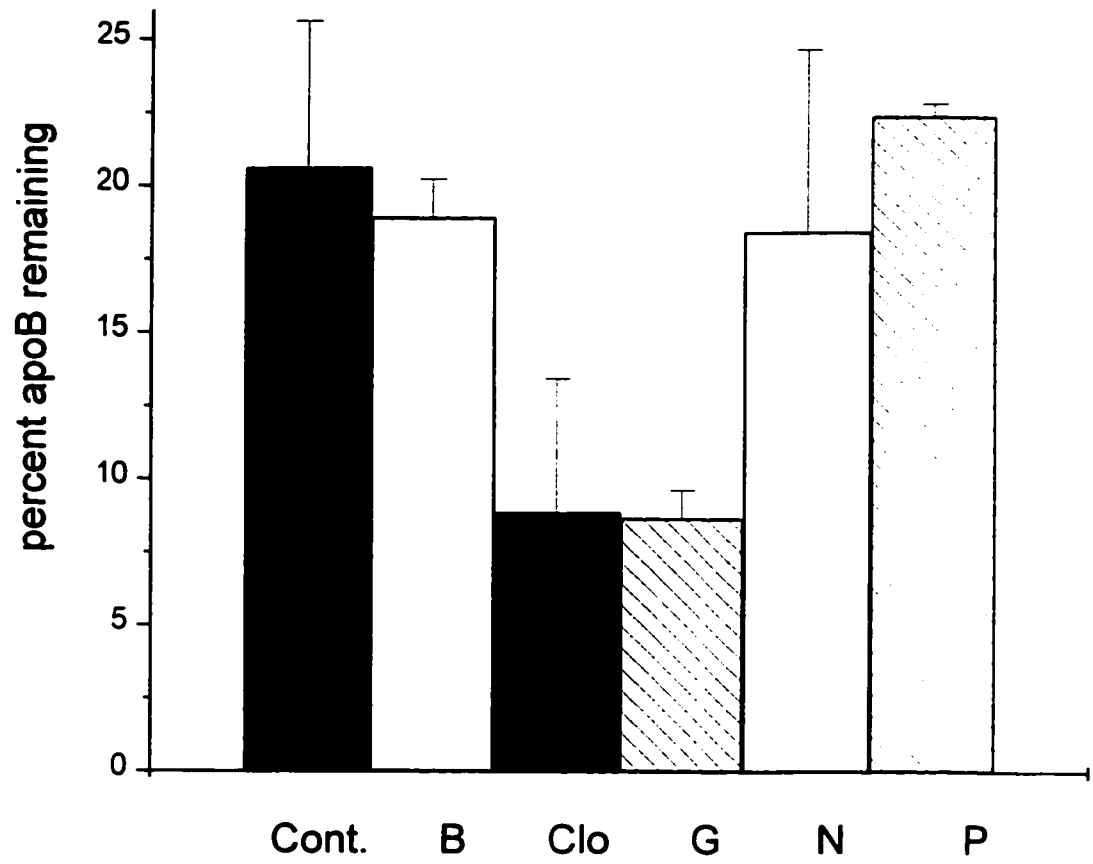
Figure 3.8

Table 3.4

**Percent ApoB Remaining in Permeabilized HepG2 Cells Treated With Nicotinic
Acid, Probucol, and Fibrate Derivatives**

Legend

HepG2 cells were treated with the same drugs and conditions as explained in Figure 3.7 legend. After the pulse and chase, cells were permeabilized with 50 µg/mL digitonin as described in Materials and Methods. After incubation in CSK buffer at 37 °C for 2-h, cells were harvested and homogenized in solubilization buffer and after centrifugation for 1 min. at 4 °C and 12000g, apoB was immunoprecipitated from supernatant as described in Materials and Methods. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer. Percent apoB remaining after 2-h in each condition was compared to the same condition at time 0 (before 2-h incubation). Analysis of data with Student's t-test and analysis of variance showed that only cells treated with gemfibrozil and clofibrate had significantly different degradation rates compared to control cells. The results are expressed as: mean ± SD.

Table 3.4

	Control cells	Bezafibrate	Clofibrate	Gemfirozil	Nicotinic acid	Probucol
% apoB remaining	20.6±5.0	18.9±1.3	8.85±4.55	8.67±0.94 *	18.4±6.2	22.34±0.40

* Significantly different from control cells as analyzed by Student's t-test ($p < 0.05$).

Figure 3.9**Effect of Gemfibrozil Treatment (120 µg/mL) on ApoB Degradation in
Permeabilized HepG2 Cells****Legend**

HepG2 cells were treated with gemfibrozil (120 µg/mL) for 4 days. Treatment was started when the cells were about 50% confluent. Cells were pulsed, chased, permeabilized and incubated in CSK as described in Materials and Methods. After harvesting, cells were homogenized in solubilization buffer and apoB was immunoprecipitated from the supernatant after centrifugation of the homogenate at 4 °C for 1min. at 12000g. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer and results were plotted as apoB remaining at each time period.

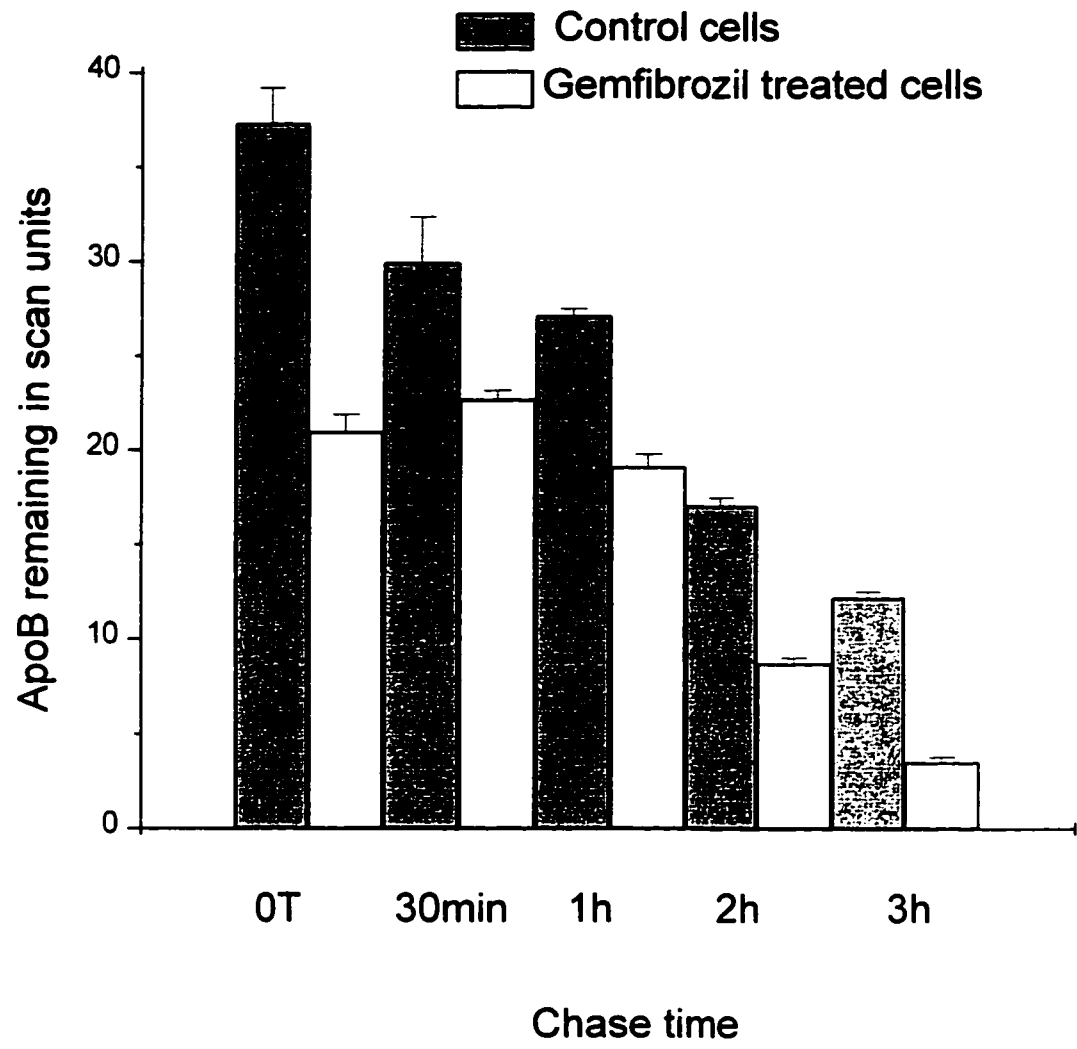
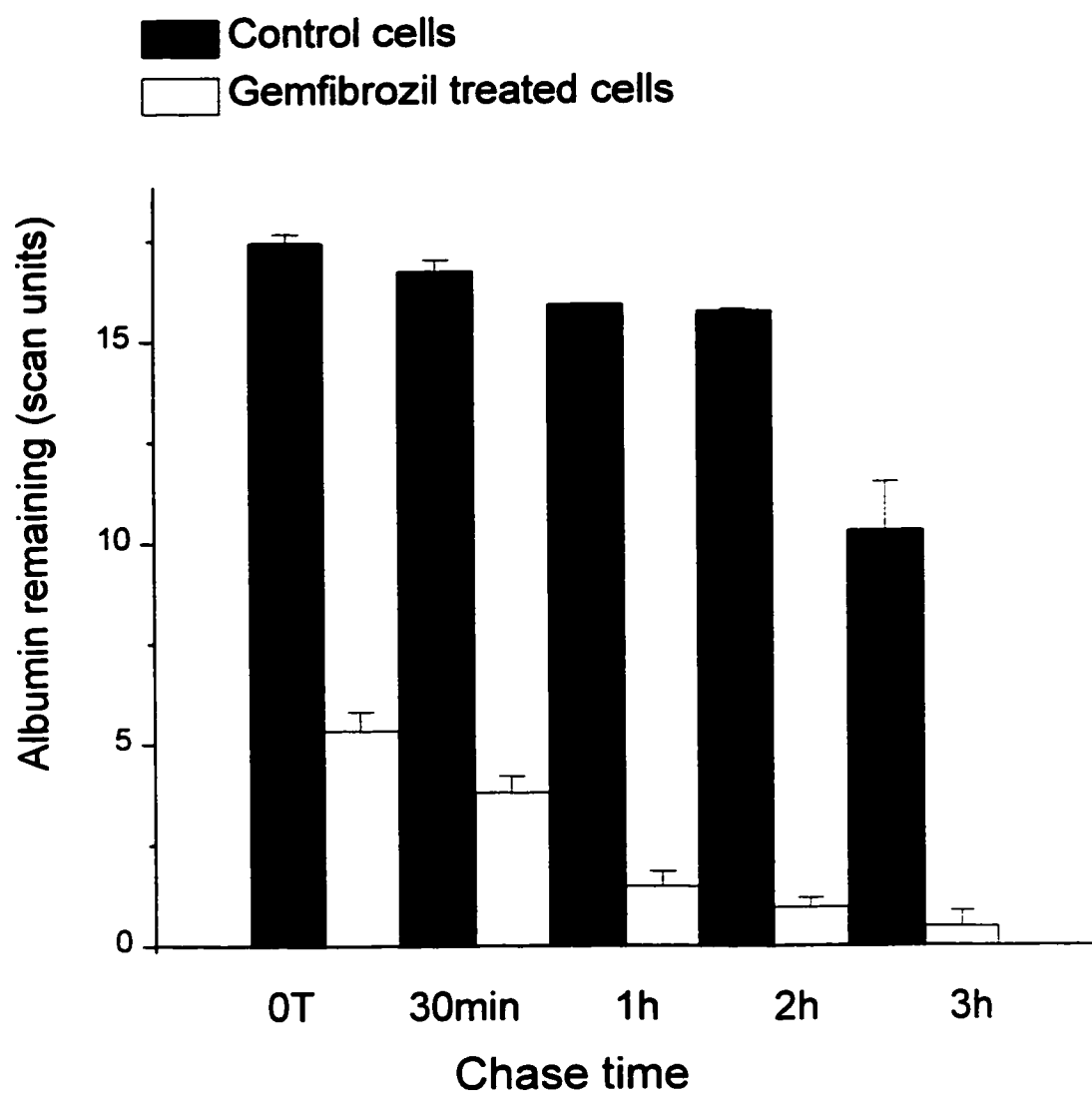
Figure 3.9

Figure 3.10**Effect of Gemfibrozil (120 µg/mL) on Albumin Turnover in HepG2 Cells****Legend**

HepG2 cells were treated with gemfibrozil (120 µg/mL) for 4 days. Treatment was started when the cells were about 50% confluent. The medium was changed every day and a new dose of drug was added to the medium. Cells were pulsed, chased, permeabilized with digitonin and incubated in CSK as described in Materials and Methods. After harvesting, cells were homogenized in solubilization buffer and albumin was immunoprecipitated from supernatant after centrifugation of the homogenate at 4 °C for 1min. at 12000g. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer and results were plotted as albumin remaining at each time period.

Figure 3.10

concentration of 120 $\mu\text{g/mL}$ was toxic to HepG2 cells and halted protein synthesis to a large extent. In a subsequent experiment, gemfibrozil was used at the concentration of 60 $\mu\text{g/mL}$ and cells were treated for 1-4 days. No significant difference was observed between control and drug treated cells in the apoB remaining after 2-h of chase (Figures 3.11 and 3.12).

Clofibrate was used at three different concentrations (50, 100, and 200 $\mu\text{g/mL}$) to treat the HepG2 cells. Cells were treated for four days and apoB was immunoprecipitated at the end of treatment period. Figure (3.13) shows the results of different concentrations of clofibrate on apoB degradation in HepG2 cells. At 50 and 100 $\mu\text{g/mL}$ clofibrate did not show any effect on apoB degradation. At 200 $\mu\text{g/mL}$ However, clofibrate caused a 50.65% decrease in apoB remaining compared to control cells.

Figure 3.11**Effect of Gemfibrozil Treatment (60 $\mu\text{g/mL}$) on ApoB Degradation in
Permeabilized HepG2 Cells****Legend**

HepG2 cells were treated with gemfibrozil (60 $\mu\text{g/mL}$) for different periods of time (1 to 4 days). Cells were pulsed, chased, permeabilized and incubated in CSK as described in Materials and Methods. After harvesting, cells were homogenized in solubilization buffer and apoB was immunoprecipitated from supernatant after centrifugation of the homogenate at 4 $^{\circ}\text{C}$ for 1min. at 12000g. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer and results were plotted as percent apoB remaining after 2-h incubation in CSK compared to time 0 (before incubation).

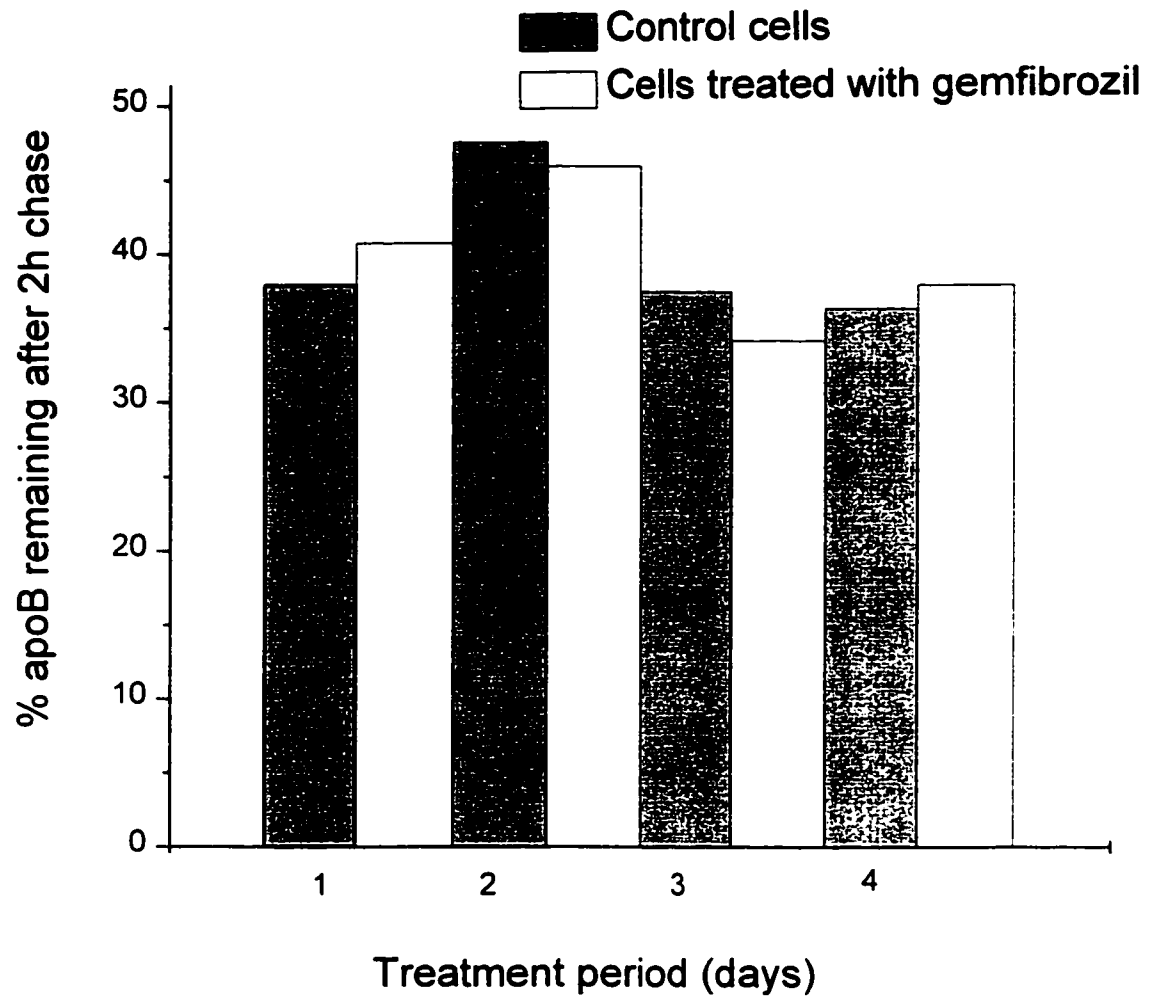
Figure 3.11

Figure 3.12**Effect of Gemfibrozil Treatment (60 $\mu\text{g/mL}$) on ApoB Degradation in
Permeabilized HepG2 Cells****Legend**

HepG2 cells were treated with gemfibrozil (60 $\mu\text{g/mL}$) for different periods of time (1 to 4 days). Cells were pulsed, chased, permeabilized and incubated in CSK as described in Materials and Methods. After harvesting, cells were homogenized in solubilization buffer and apoB was immunoprecipitated from supernatant after centrifugation of the homogenate at 4 $^{\circ}\text{C}$ for 1min. at 12000g. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. The bands corresponding to apoB were cut out of the gels and counted as described in Materials and Methods. Results were plotted as apoB remaining at each time period under different conditions.

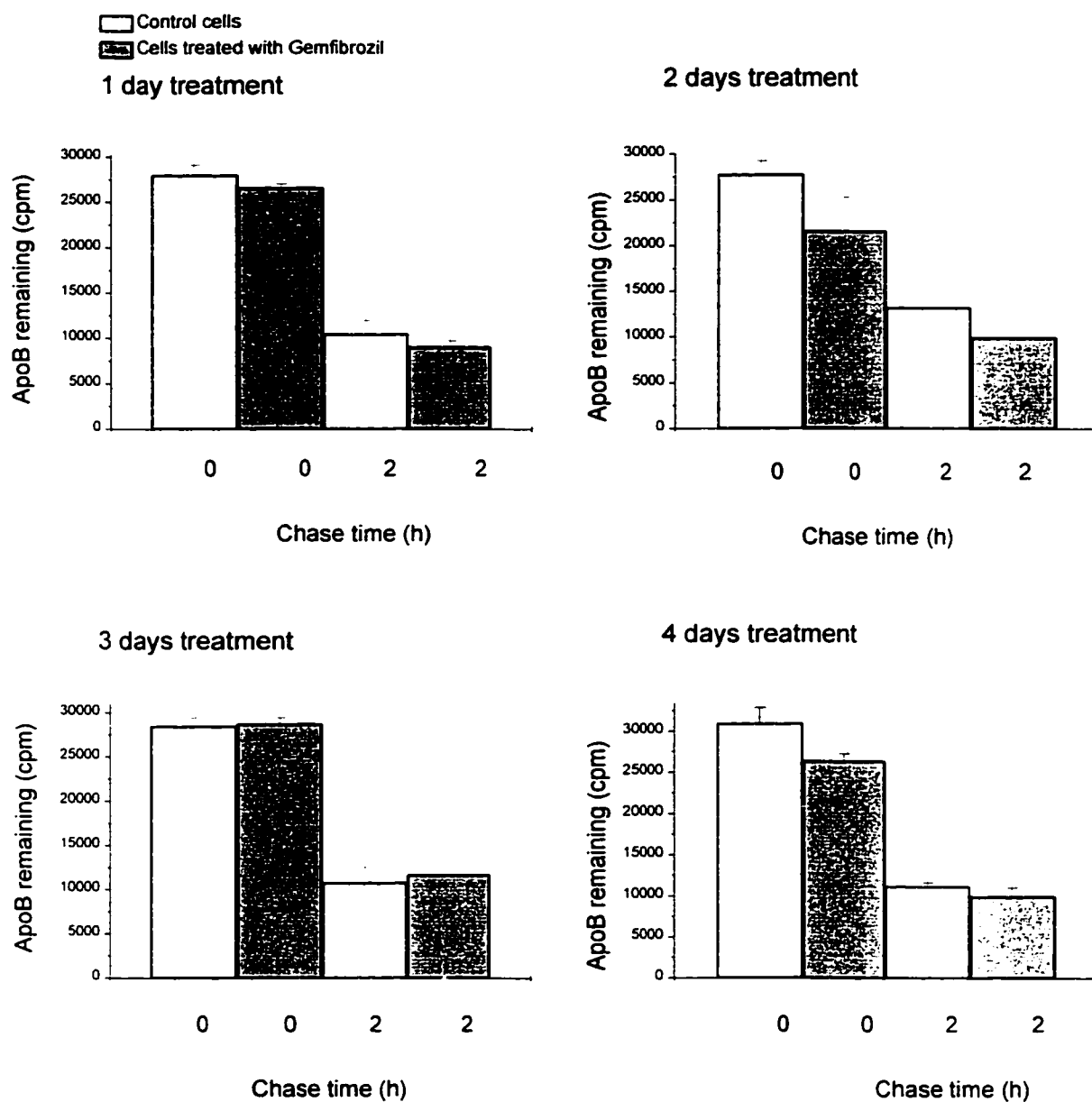
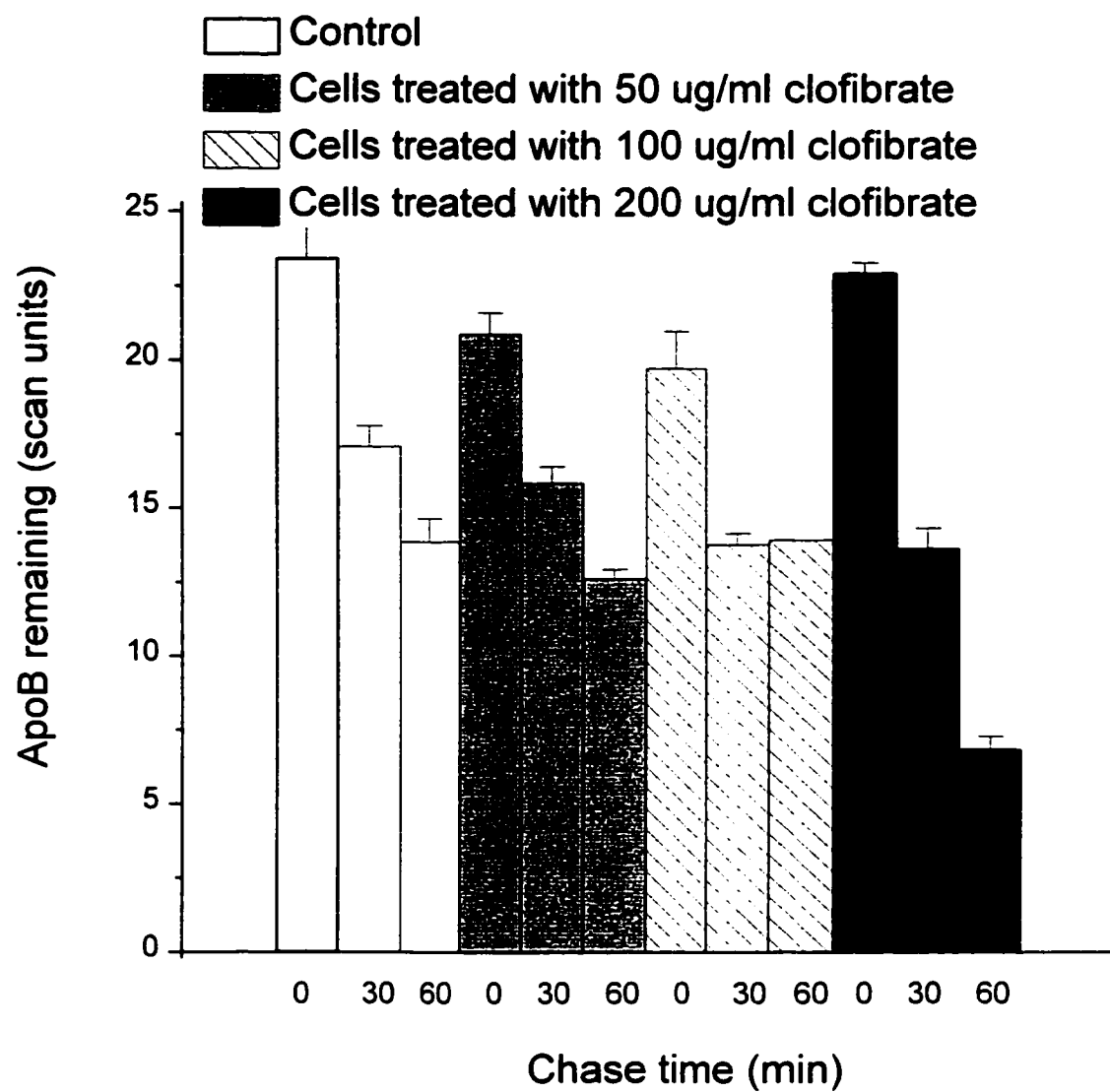
Figure 3.12

Figure 3.13**Effect of Clofibrate on ApoB Degradation in Permeabilized HepG2 Cells****Legend**

HepG2 cells were treated with clofibrate at different concentrations (50, 100, and 200 $\mu\text{g/mL}$) for 4 days. Treatment was started when the cells were about 50-60% confluent. Cells were pulsed, chased, permeabilized and incubated in CSK as described in Materials and Methods. After harvesting, cells were homogenized in solubilization buffer and apoB was immunoprecipitated from supernatant after centrifugation of the homogenate at 4 °C for 1min. at 12000g. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer and results were plotted as apoB remaining at each time period under different conditions.

Figure 3.13

3.3.2 Effect of HMG-CoA Reductase Inhibitor, Atorvastatin on ApoB Turnover in HepG2 Cells

3.3.2.1 Effect of Atorvastatin on ApoB Secretion by HepG2 Cells

Initially the effect of atorvastatin on the secretion of apoB was determined in intact HepG2 cells that were treated overnight with a single dose of the drug in the presence or absence of oleate. Cells were either pulsed for 2-h continuously (Figure 3.14A) or pulsed for 10 min. and chased in complete medium for 2-h (Figure 3.14B). As expected, there was a significant (2.28-fold) stimulation of apoB secretion in cells incubated with oleate (13.19 ± 0.87 , $n=4$) when compared with control cells (5.79 ± 0.37 , $n=4$). However, incubation with both oleate and atorvastatin resulted in a lower stimulation of apoB secretion (10.46 ± 0.43 , $n=4$). There was a significant decrease ($p < 0.005$) in the amount of immunoprecipitable apoB in cells treated with both the drug and oleate, when compared with that in cells treated with oleate alone. Treatment with atorvastatin appeared therefore to partially abolish the stimulatory effect of oleate on apoB secretion.

3.3.2.2 Effect of Atorvastatin on ApoB Degradation in HepG2 Cells

To determine whether the effect of atorvastatin was in part at the level of apoB degradation, a pulse-chase experiment was performed in intact HepG2 cells (Figure 3.15). Cells were pretreated with oleate and with or without a single dose of the inhibitor. Cells were pulsed for 10 min., and then chased for 2-h. ApoB was immunoprecipitated at both 0 time and 2-h, and the extent of apoB degradation was estimated by determining the percent apoB remaining after 2-h chase. As expected, oleate-treatment resulted in a significant

Figure 3.14**Effect of Atorvastatin on ApoB Secretion by HepG2 Cells****Legend**

Monolayer HepG2 cells were treated overnight with a single dose of atorvastatin (10 μ M) in the presence or absence of oleate. Cells were either pulsed for 2-h continuously (A) or pulsed for 10 min. and chased in complete medium for 2-h (B). At the end of incubation period, medium was collected and centrifuged at 5000 rpm to pellet any floating cells. Medium was then carefully removed and mixed with an equal volume of solubilization buffer. ApoB was then immunoprecipitated as described in Materials and Methods. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer and results were plotted as apoB secreted under different conditions.

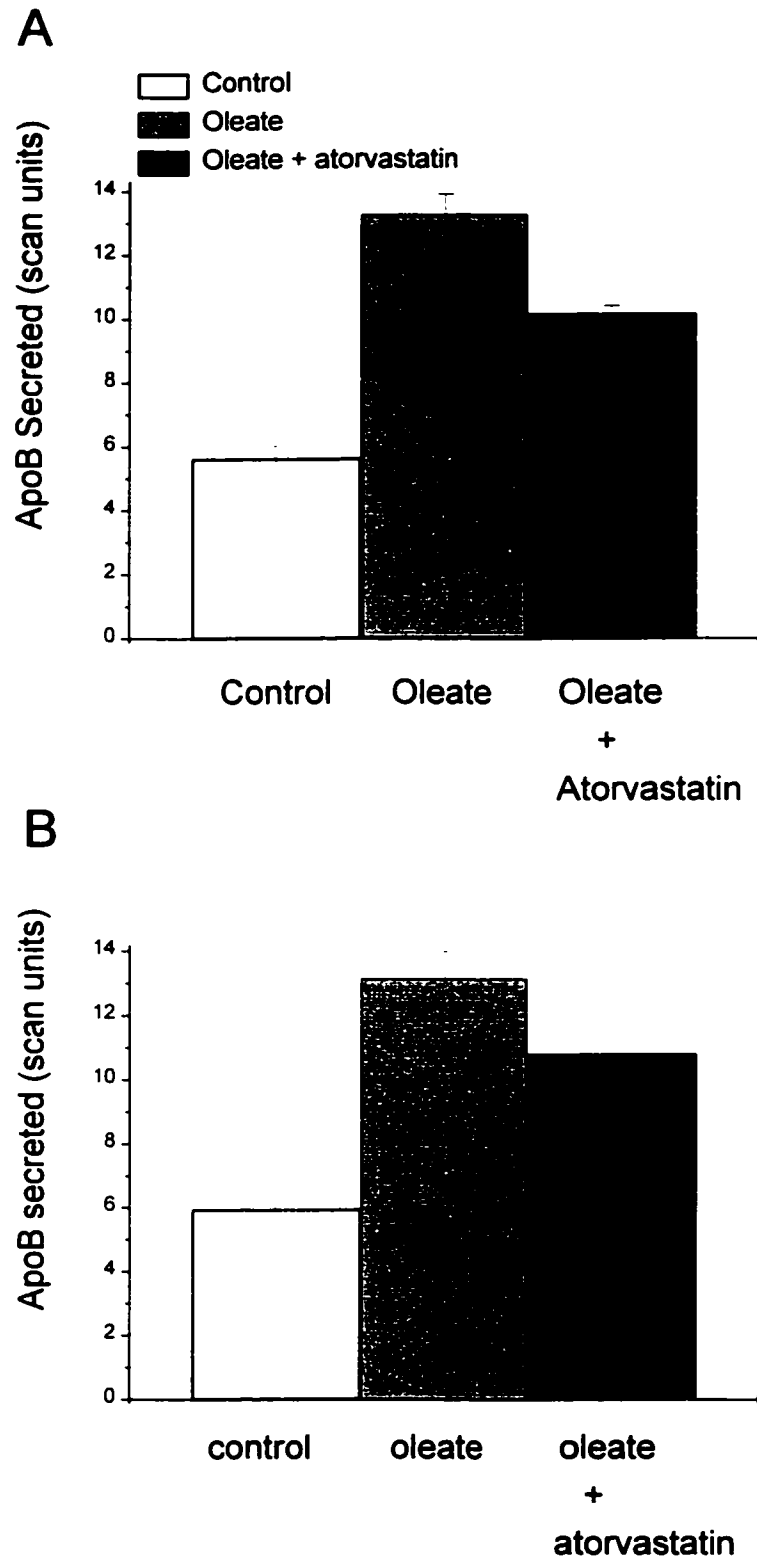
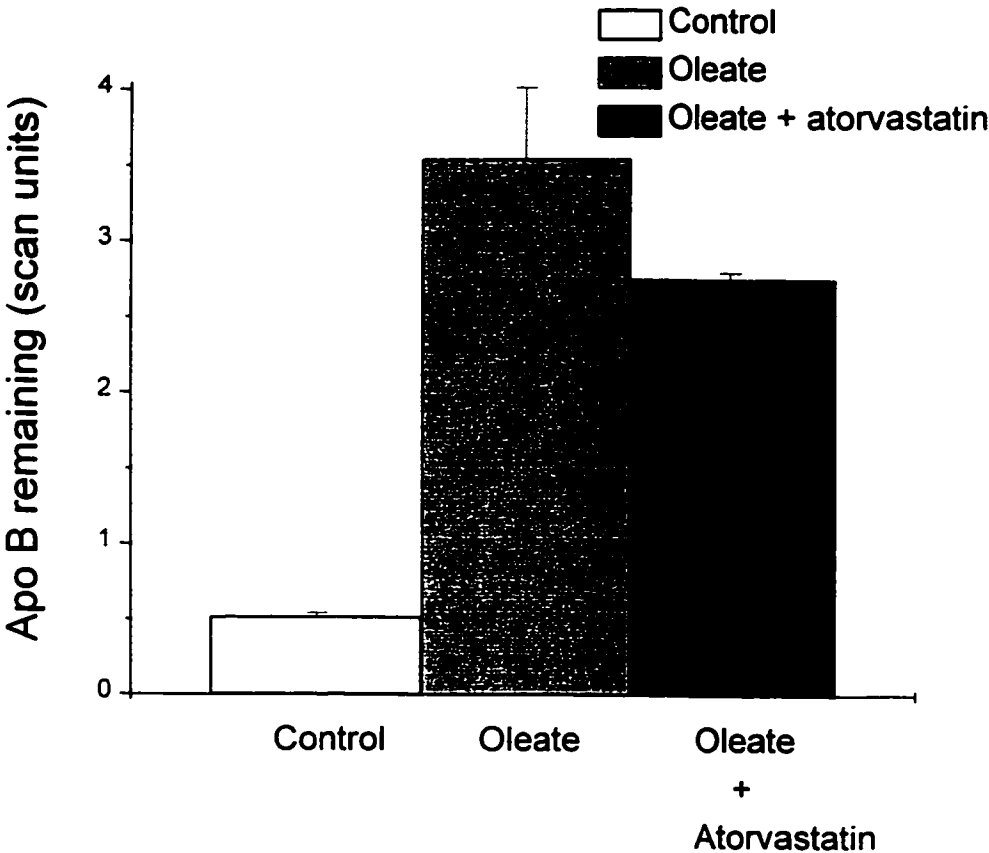
Figure 3.14

Figure 3.15**Effect of Atorvastatin on ApoB Degradation in Intact HepG2 Cells****Legend**

HepG2 cells were treated overnight with oleate in the presence (10 μ M) or absence of atorvastatin. Cells were pulsed for 10 min. and chased in complete medium for 2-h and then both medium and cells were harvested. ApoB was immunoprecipitated at 0 and 2-h of chase. Cells were homogenized in solubilization buffer and centrifuged at 4 $^{\circ}$ C for 1 min. at 12000g. ApoB was immunoprecipitated from supernatant. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer. The results were then plotted as apoB remaining under different conditions.

Figure 3.15

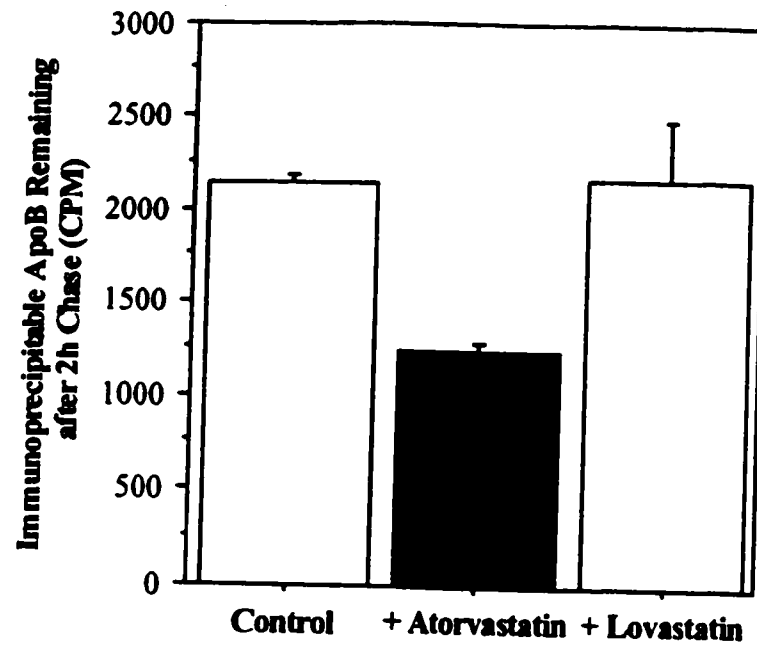


elevation (4.05-fold) in apoB remaining in cells chased for 2-h when compared with that in control cells. This suggests that oleate treatment protected apoB from degradation. In cells treated with oleate + atorvastatin, there was a significant drop (by 25.4%) in the amount of immunoprecipitable apoB after the chase, when compared to oleate-treated cells. Thus, percent apoB remaining in drug treated cells was $26.18 \pm 0.63\%$ compared to $35.08 \pm 3.31\%$ in control cells.

To further investigate the effect of atorvastatin on apoB degradation, several experiments were carried out in cells permeabilized with digitonin. We had previously used permeabilized HepG2 cells to investigate apoB degradation and have shown the usefulness of this system in studying the degradation process (Adeli, 1994). Intact HepG2 cells were first treated overnight with 10 μM atorvastatin, pulsed with [^{35}S]methionine, briefly chased, and then permeabilized with digitonin. Permeabilized cells were incubated in buffer and degradation was monitored by immunoprecipitating apoB before and after a 2-h chase. Figure (3.16) shows the amount of apoB radioactivity recovered by immunoprecipitation after the chase in control cells as well as in cells pretreated with atorvastatin or lovastatin. Preincubation with lovastatin did not appear to affect the stability of apoB in permeabilized HepG2 cells, as the amount of apoB signal recovered was comparable to that in control cells. However, atorvastatin-treated cells contained significantly lower amounts of immunoprecipitable apoB at the end of the 2-h chase, suggesting that atorvastatin reduced apoB stability and increased its intracellular degradation. There was a 42% decrease (average of two experiments) in immunoprecipitable apoB remaining in atorvastatin-treated cells.

Figure 3.16**Effect of Atorvastatin and Lovastatin on ApoB Degradation in Permeabilized****HepG2 Cells****Legend**

HepG2 cells were treated overnight with atorvastatin or lovastatin (10 μ M) in the presence of oleate (0.3 mM). Cells were pulsed, chased, permeabilized and incubated in CSK for 2-h as described in Materials and Methods. After harvesting, cells were homogenized in solubilization buffer and apoB was immunoprecipitated from supernatant after centrifugation of the homogenate at 4 $^{\circ}$ C for 1min. at 12000g. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. The bands corresponding to apoB were cut out of the gels and counted as described in Materials and Methods. Results were plotted as apoB remaining at each time period under different conditions.

Figure 3.16

In a separate experiment (Figure 3.17), HepG2 cells were treated with multiple doses of atorvastatin at two different concentrations (10 μ M and 20 μ M). Drug treatment at 20 μ M every 3 h (4 doses) decreased apoB remaining in the cells by 47%, compared to a 23.6% decrease with multiple doses of 10 μ M atorvastatin. Atorvastatin-mediated stimulation of apoB degradation was therefore concentration-dependent.

Effect of atorvastatin on apoB degradation was also investigated in the presence of LDL. HepG2 cells were preincubated with LDL only or with LDL plus atorvastatin. ApoB degradation was then monitored after pulse, chase, and permeabilization of the cells as described. As shown in Figure 3.18, LDL treatment of HepG2 cells reduced the amount of apoB remaining by 40.3%. Treatment with multiple doses of atorvastatin (10 μ M doses every 3 h x 4) decreased apoB remaining in LDL-treated cells by an additional 26%, suggesting a stimulation of apoB degradation. The effect of atorvastatin on apoB degradation was also determined in cells treated for 6 h vs 20 h. Cells pretreated for either 6 h or 20 h were pulsed, chased for 10 min., and then permeabilized with digitonin. Permeabilized cells were incubated in buffer to monitor apoB degradation. As depicted in Figure 3.19, treatment for either 6 h or 20 h resulted in a significant drop in apoB radioactivity after a 2-h chase, suggesting an enhancement of apoB degradation. Drug treatment for both time periods appeared to have similar effects on apoB stability, indicating that atorvastatin elicited its effect within 6h of pre-incubation.

Figure 3.17**Effect of Multiple Doses of Atorvastatin on ApoB Degradation in HepG2 Cells****Legend**

HepG2 cells were treated overnight with multiple doses of atorvastatin (4 doses, every 3h) at two different concentrations (10 and 20 μ M). Cells were pulsed, chased, permeabilized and incubated in CSK as described in Materials and Methods. After harvesting, cells were homogenized in solubilization buffer and apoB was immunoprecipitated from supernatant after centrifugation of the homogenate at 4 $^{\circ}$ C for 1min. at 12000g. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer and results were plotted as apoB remaining at each time period.

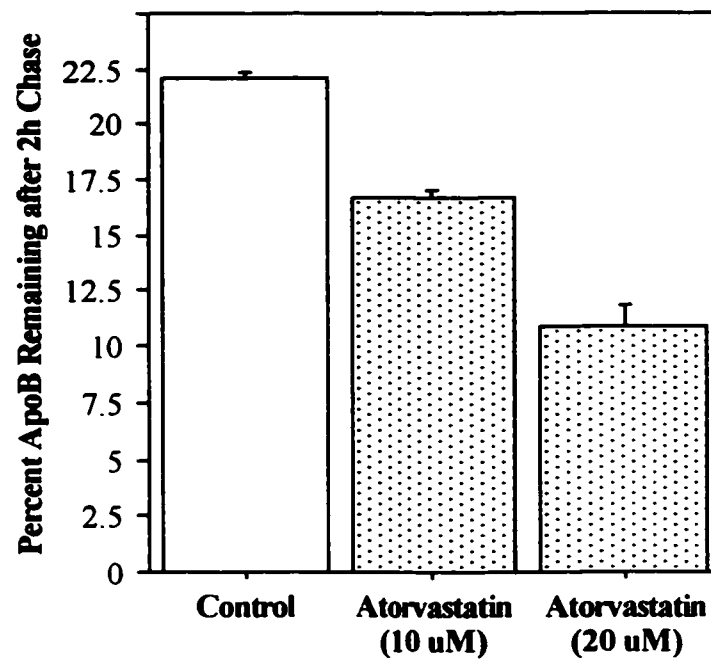
Figure 3.17

Figure 3.18**Effect of Atorvastatin on ApoB Degradation in HepG2 Cells, in the Presence of LDL****Legend**

HepG2 cells were treated overnight with LDL (100 $\mu\text{g/mL}$ of LDL protein) in the presence or absence of atorvastatin (10 μM). Cells were pulsed, chased, permeabilized and incubated in CSK as described in Materials and Methods. After harvesting, cells were homogenized in solubilization buffer and apoB was immunoprecipitated from supernatant after centrifugation of the homogenate at 4 $^{\circ}\text{C}$ for 1min. at 12000g. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer and results were plotted as apoB remaining at each time period.

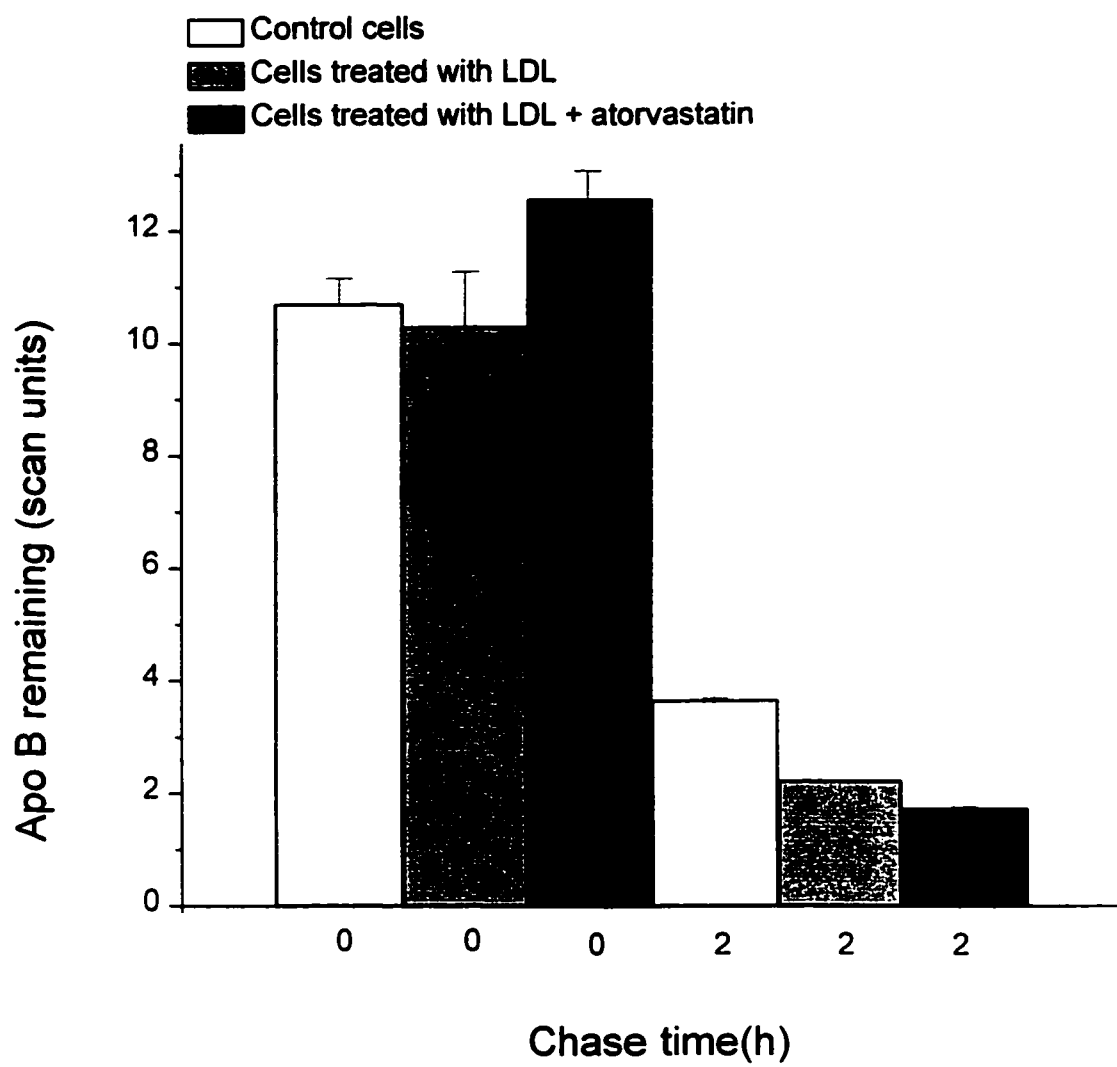
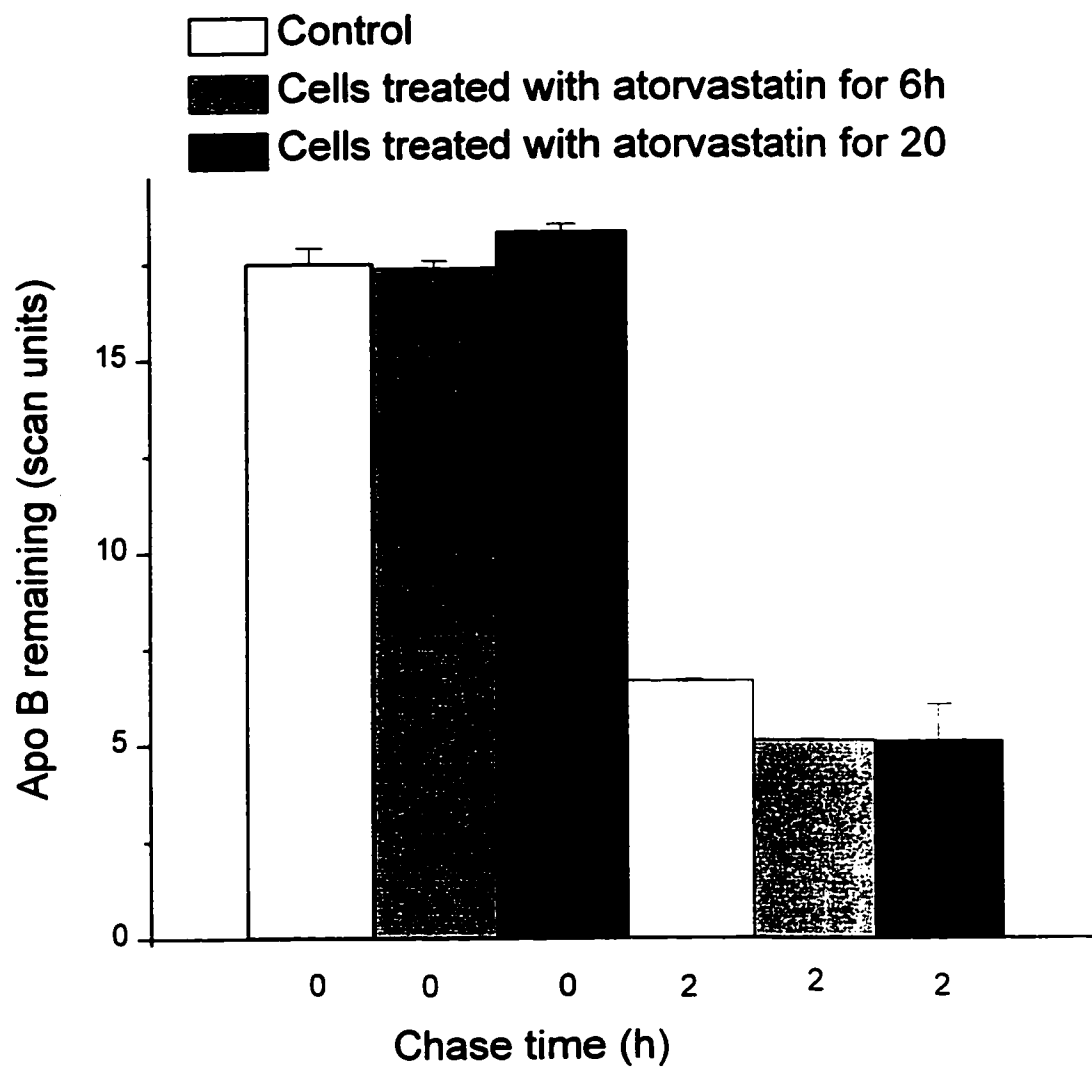
Figure 3.18

Figure 3.19**Degradation of ApoB in HepG2 Cells Treated with Atorvastatin for Different Periods of Time****Legend**

HepG2 cells were treated with a single dose of atorvastatin (10 μ M) for 6 and 12-h time periods. Cells were pulsed, chased, permeabilized and incubated in CSK as described in Materials and Methods. After harvesting, cells were homogenized in solubilization buffer and apoB was immunoprecipitated from supernatant after centrifugation of the homogenate at 4 °C for 1 min. at 12000g. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer and results were plotted as apoB remaining at each time period.

Figure 3.19

The rate of apoB degradation in permeabilized HepG2 cells was also monitored in both control cells and atorvastatin-treated cells (Figure 3.20). Treatment with the inhibitor appeared to accelerate the degradation rate of apoB. Over the first hour of chase, the amount of apoB radioactivity remaining was $72.75\% \pm 3.93$ (n=3) in control cells and 49.61 ± 1.96 (n=3) in drug treated cells. The rate of degradation during the first hour of chase was approximately two fold greater in atorvastatin-treated cells (based on regression analysis, the slope of apoB loss in control cells was -27.2 compared to -50.4 for drug treated cells). The rise in the apoB degradation rate also correlated well with the data presented above indicating a 24.7% decline in the total apoB radioactivity after a 2-h chase period.

3.3.2.3 Effect of Atorvastatin on the Degradation of Different ApoB Pools in HepG2 Cells.

Effect of atorvastatin on the degradation of different apoB pools in HepG2 cells was also investigated. After a 15-min. pulse and 20-min. chase period, cells were permeabilized with digitonin and then chased in CSK buffer for 0 or 2-hours. Microsomal membrane and luminal fractions were then separated as described in Materials and Methods, and apoB was immunoprecipitated. When total apoB radioactivity accumulated in the ER lumen were compared between control cells and drug-treated cells, there was a significant decline with atorvastatin treatment after the 2-h chase (Figure 3.21A). Similarly, the apoB immunoprecipitated from the membrane fraction (Figure 3.21B) was significantly reduced after the chase in drug-treated cells compared to control cells. despite the observation that approximately similar amount of apoB radioactivity was associated with the membrane at 0 time (before the chase). This

Figure 3.20**Effect of Atorvastatin on the Rate of ApoB Degradation in HepG2 Cells****Legend**

HepG2 cells were treated overnight with atorvastatin (10 μ M). Cells were pulsed, chased, permeabilized and incubated in CSK for 0, 1, and 2-h as described in Materials and Methods. After harvesting, cells were homogenized in solubilization buffer and apoB was immunoprecipitated from supernatant after centrifugation of the homogenate at 4 $^{\circ}$ C for 1min. at 12000g. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. X-ray films were then scanned by a Bio-Rad GS-670 densitometer and results were plotted as apoB remaining at each time period.

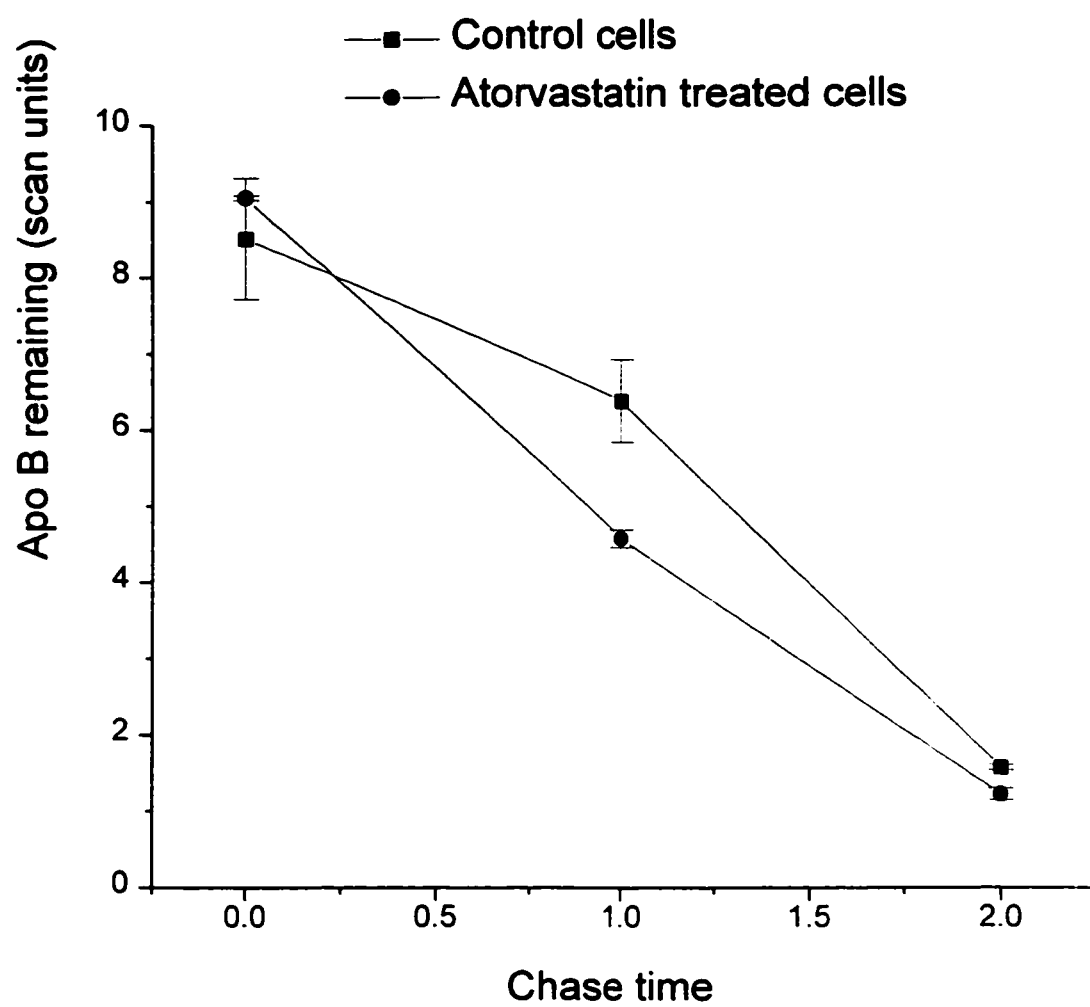
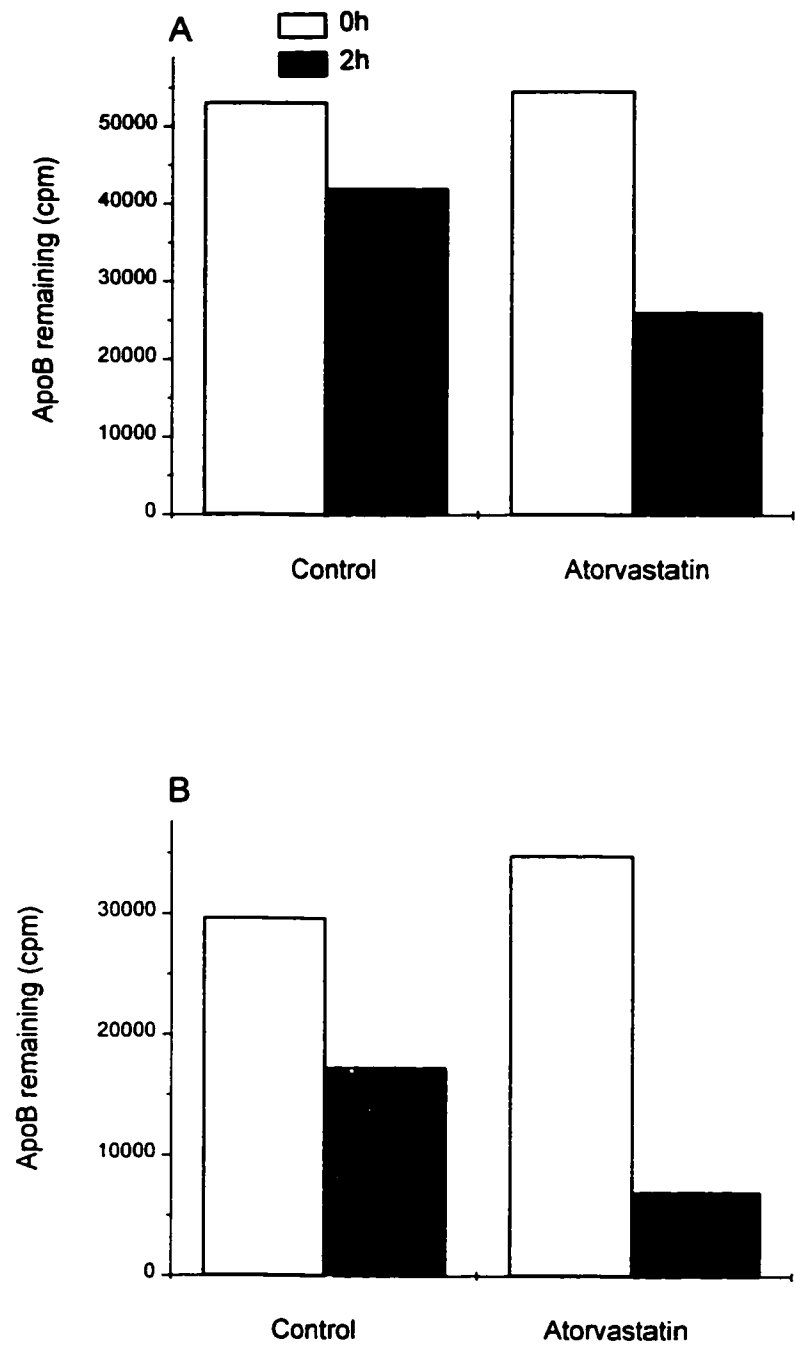
Figure 3.20

Figure 3.21**Effect of Atorvastatin on Degradation of ApoB in the Membrane and Lumen of ER
in HepG2 Cells****Legend**

HepG2 cells were treated with a single dose of atorvastatin (10 μ M) overnight. Cells were pulsed, chased, permeabilized and incubated in CSK as described in Materials and Methods. Luminal (A) and microsomal membrane fractions (B) were then separated by ultracentrifugation as described in Materials and Methods. ApoB was then immunoprecipitated from luminal (A) and membrane (B) fractions. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. The bands corresponding to apoB were cut out of the gels and counted as described in Materials and Methods. Results were plotted as apoB remaining at each time period.

Figure 3.21



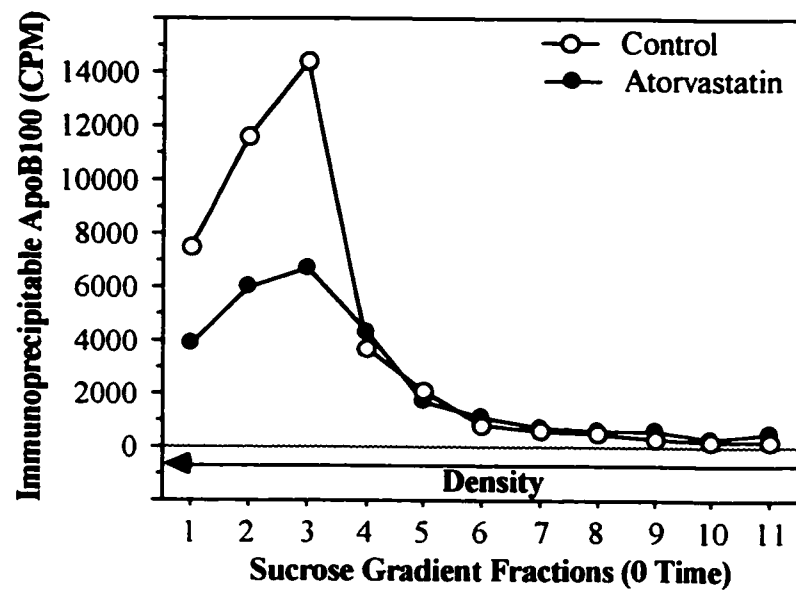
suggests that membrane-associated apoB was less stable and degraded faster in drug-treated HepG2 cells.

To further investigate the effect of atorvastatin on the degradation of different apoB species in the ER lumen, HepG2 cells were pulsed, chased for 10 min., and then permeabilized with digitonin. Permeabilized cells were incubated for 2-h in CSK buffer and then subjected to subcellular fractionation as described in Materials and Methods. Luminal lipoprotein particles were isolated from total microsomes and then analyzed by sucrose-gradient ultracentrifugation and immunoprecipitation. As depicted in Figure 3.22, there was a significantly lower amount of apoB-containing lipoprotein particles in fractions 1-5 in atorvastatin-treated cells. These results suggest that the HDL-like particles (fractions 1-5) are more susceptible to degradation in atorvastatin treated cells as compared to controls.

Taken together, these experiments support an effect of atorvastatin on enhancing intracellular apoB degradation, and reducing the formation and accumulation of apoB containing lipoprotein particles in the lumen of the secretory pathway in HepG2 cells.

Figure 3.22**Effect of Atorvastatin on Accumulation and Stability of Different ApoB Species in the Lumen of ER in HepG2 Cells****Legend**

HepG2 cells were treated with a single dose of atorvastatin (10 μ M) overnight. Cells were pulsed, chased, permeabilized and incubated in CSK as described in Materials and Methods. Cells were then harvested and homogenized after 2-h chase. Following separation of microsomal membranes and luminal compartments, the luminal part was further fractionated on a discontinuous sucrose gradient according to Materials and Methods. ApoB was then immunoprecipitated from each fraction and immunoprecipitates were analyzed by SDS-PAGE and fluorography. The bands corresponding to apoB were cut out of the gels and counted as described in Materials and Methods. Fractions 2-5 represent high density apoB lipoprotein particles (HDL-like-apoB, peak density 1.065-1.117 g/mL), and fractions 6-11 represent the lower density apoB lipoprotein particles (LDL/VLDL-like-apoB, peak density 1.011-1.045 g/mL) (Boren *et al.*, 1990; Boren *et al.*, 1992).

Figure 3.22

3.4 Studies on Mechanisms of Intracellular ApoB Degradation

In order to study the fate of apoB bound to the ER membrane and the luminal apoB, HepG2 cells were pulsed, chased, and permeabilized according to the procedures described. Cells were then homogenized and cell debris was separated by centrifugation. The microsomal contents were then released by sodium carbonate treatment (Boren *et al.*, 1992). Microsomal membranes and luminal contents were then separated by ultracentrifugation. ApoB, albumin, and α_1 -antitrypsin were then immunoprecipitated from each fraction according to procedures described in Materials and Methods. Figure 3.23 shows the immunoprecipitation of albumin and α_1 -antitrypsin from membrane and luminal fractions. Two conclusions could be made from the results. First, isolation of membrane and luminal fractions must have been performed efficiently as there was negligible amounts of albumin and α_1 -antitrypsin found in the membrane fraction. As was expected for a secretory protein, the majority of albumin ($96.7 \pm 5.0\%$ mean \pm SD $n=3$) was recovered from the luminal content, while only a minor fraction of albumin ($3.3 \pm 0.5\%$) was recovered from the membrane fraction. This was also the case for α_1 -antitrypsin. Secondly, the degrading system was specific for apoB, since neither albumin nor α_1 -antitrypsin were to any significant degree degraded during the 2-h chase. The recovery of albumin and α_1 -antitrypsin after 2-h of chase were $103.1 \pm 0.6\%$, and $85.6 \pm 16.2\%$ (mean \pm SD $n=3$) of their corresponding amount at 0h, respectively.

Figure 3.23**Turnover of Albumin and α 1-Antitrypsin in HepG2 Cells****Legend**

HepG2 cells were pulsed, chased, and incubated in CSK for different periods of time. Microsomal membrane and luminal fractions were then separated by ultracentrifugation as described in Materials and Methods. Albumin (A) and α 1-antitrypsin (B) were then immunoprecipitated from membrane and luminal fractions. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. The bands corresponding to albumin and α 1-antitrypsin were cut out of the gels and counted as described in Materials and Methods. Percent apoB remaining was calculated based on the amount of apoB present at time 0.

Figure 3.23

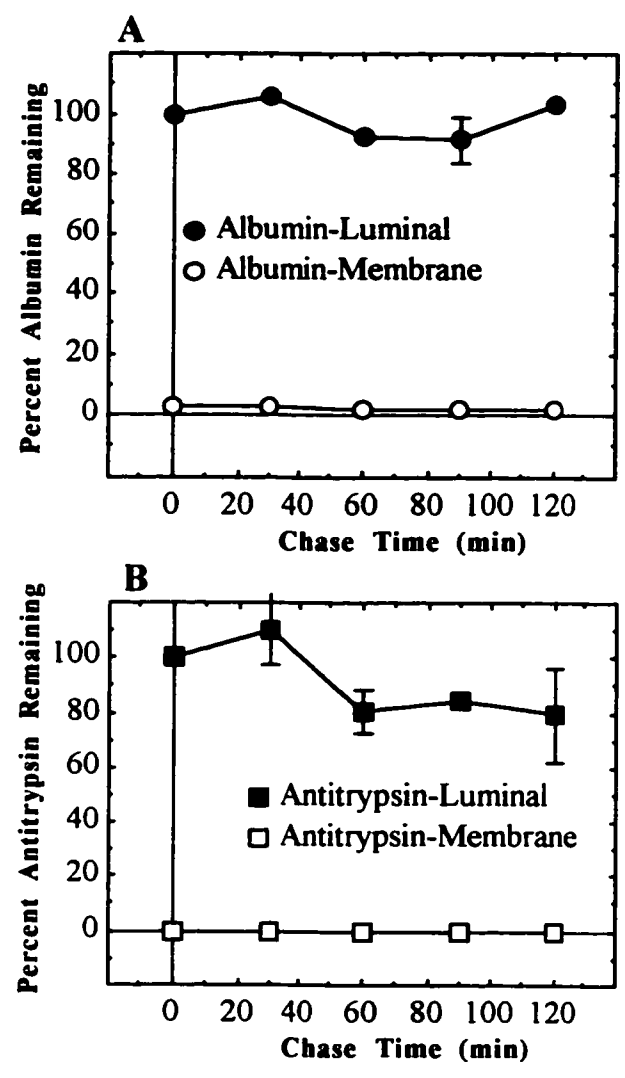


Figure 3.24 represents the results obtained from immunoprecipitation of apoB from membrane and luminal fractions. Although at 0h comparable amounts of apoB were recovered from both membrane and lumen of the ER, after 2-h of chase the apoB remaining in the membrane fraction was considerably lower than that in the lumen suggesting a higher extent of apoB degradation in the membrane.

The rate of apoB degradation was also compared in the membrane and luminal fractions (Figure 3.25). The results showed that pulse-labeled apoB100 that was associated with the membrane fraction was rapidly and almost totally degraded over the 2-h chase. In comparison, the [^{35}S]-labeled apoB detected in the luminal fraction of the microsomes was more stable, although it was also significantly degraded over the 2-h chase. Degradation rates were particularly different over the first hour of chase with the degradation rate of membrane-bound apoB being much faster. Over 80% of membrane-associated apoB was lost during the first hour of chase. In contrast, the luminal apoB pool was degraded at a slower rate with just over 40% degraded after 1-h chase. The data indicate that both the luminal and membrane-bound pools of apoB were subjected to intracellular degradation, However, the degradation of the two pools occurred at different rates.

Concomitant with the disappearance of the apoB100 from the microsomal lumen. degradation intermediates including the earlier identified 70 kDa fragment (Adeli, 1994). appeared (Figure 3.26). The 70 kDa fragment was mainly recovered from luminal fractions with the HDL density range. On the contrary, we could only find trace amounts of the 70 kDa fragment in association with the membrane fraction. Results from a pulse-

Figure 3.24**Degradation of Luminal and Membrane Bound ApoB in the ER of HepG2 Cells****Legend**

HepG2 cells were pulsed, chased, permeabilized and incubated in CSK as described in Materials and Methods. Microsomal membrane and luminal fractions were then separated by ultracentrifugation as described in Materials and Methods. ApoB was then immunoprecipitated from membrane and luminal fractions. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. The bands corresponding to apoB were cut out of the gels and counted as described in Materials and Methods. Percent apoB remaining was calculated based on the amount of apoB present at time 0 in each fraction

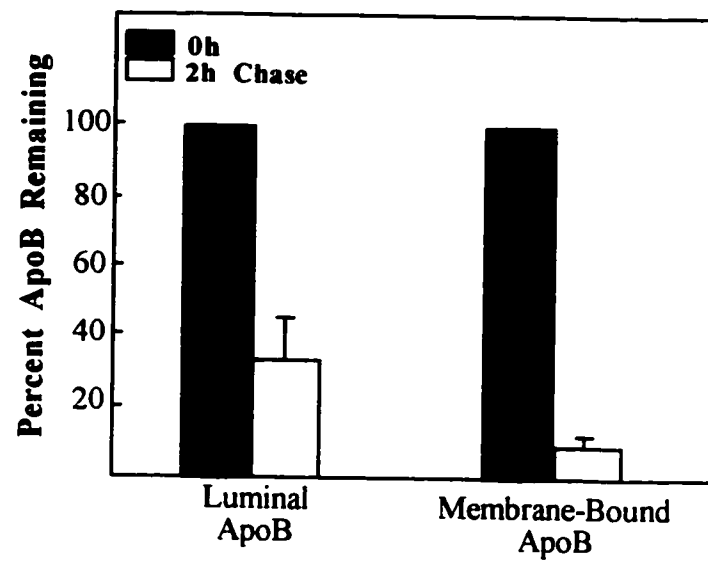
Figure 3.24

Figure 3.25**Rate of Luminal and Membrane-Bound ApoB Degradation in the ER of HepG2****Cells****Legend**

HepG2 cells were pulsed, chased, permeabilized and incubated in CSK for different periods of time. Microsomal membrane and luminal fractions were then separated by ultracentrifugation as described in Materials and Methods. ApoB was then immunoprecipitated from membrane and luminal fractions. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. The bands corresponding to apoB were cut out of the gels and counted as described in Materials and Methods. Percent apoB remaining was calculated based on the amount of apoB present at time 0 in each fraction.

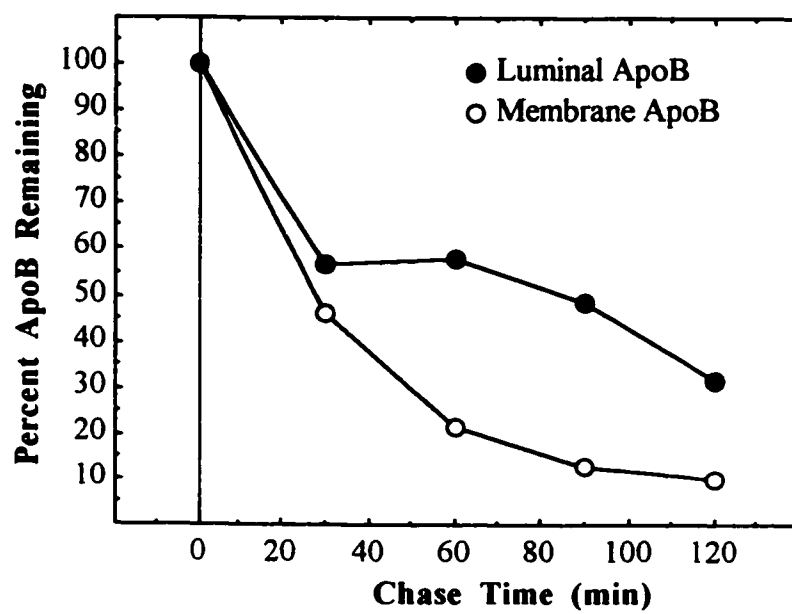
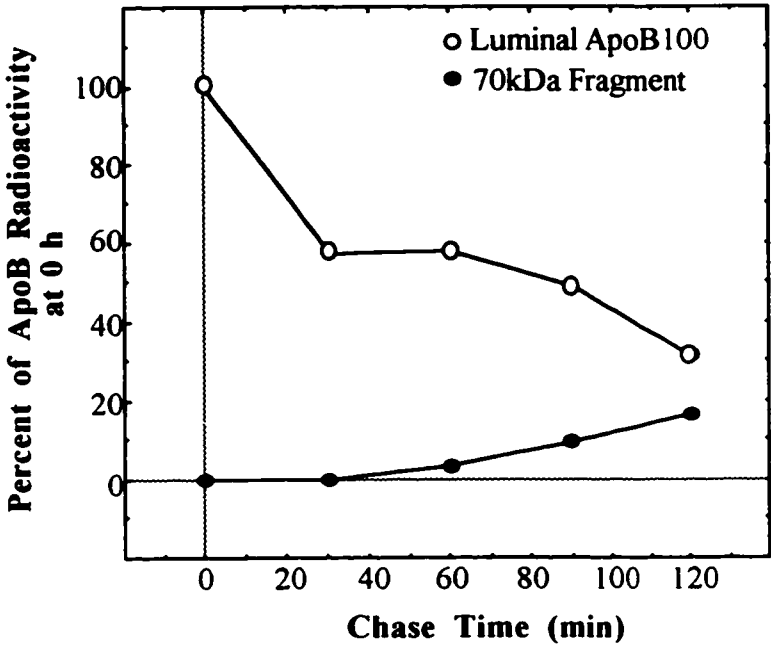
Figure 3.25

Figure 3.26**Generation of a 70 kDa Fragment from Degradation of ApoB in the ER Lumen of HepG2 Cells****Legend**

HepG2 cells were pulsed, chased, permeabilized and incubated in CSK for different periods of time. Cells were then solubilized and microsomal membrane and luminal fractions were separated by ultracentrifugation as described in Materials and Methods. ApoB was then immunoprecipitated from membrane and luminal fractions. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. The bands corresponding to intact apoB and the 70 kDa fragment were cut out of the gels and their radioactivity was counted as described in Materials and Methods. Percent apoB remaining was calculated based on the amount of intact apoB present at 0h.

Figure 3.26



chase experiment (Figure 3.27) indicated that the 70 kDa fragment accumulated in the microsomal lumen when apoB100 was degraded.

ALLN slowed down the degradation rate of luminal apoB considerably (Figure 3.28). At 30 min. of chase, control cells had retained only 56.9% of luminal apoB radioactivity at 0 time, whereas ALLN-treated cells had retained 83.8% of luminal apoB. Although some of the luminal apoB was still degraded in ALLN-treated cells, the total apoB remaining was considerably higher in ALLN-treated cells compared to that in control cells. Interestingly, treatment with ALLN appeared to abolish the generation of the 70 kDa fragment in the luminal contents of permeabilized cells (Figure 3.27).

In contrast the rate of the disappearance of the membrane-associated apoB100 was not influenced by ALLN (Figure 3.28). Thus the percent membrane-bound apoB remaining after 2-h chase were $9.0 \pm 2.5\%$ (– ALLN, mean \pm SD n=8) and $7.3 \pm 0.4\%$ (– ALLN, mean \pm SD n=3) (not statistically different)

Figure 3.27**Inhibition of Luminal ApoB Degradation by ALLN****Legend**

HepG2 cells were pulsed, chased, permeabilized, and incubated in CSK in the absence or presence of ALLN (40 $\mu\text{g/mL}$). Cells were then solubilized and microsome and luminal fractions were separated by ultracentrifugation as described in Materials and Methods. ApoB was then immunoprecipitated from luminal fraction. The immunoprecipitates were analyzed by SDS-PAGE and fluorography.

Figure 3.27

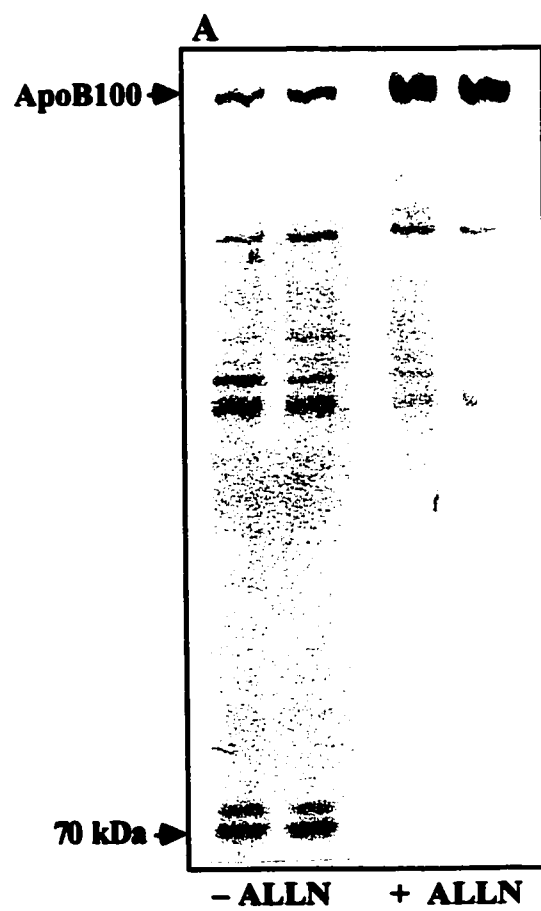
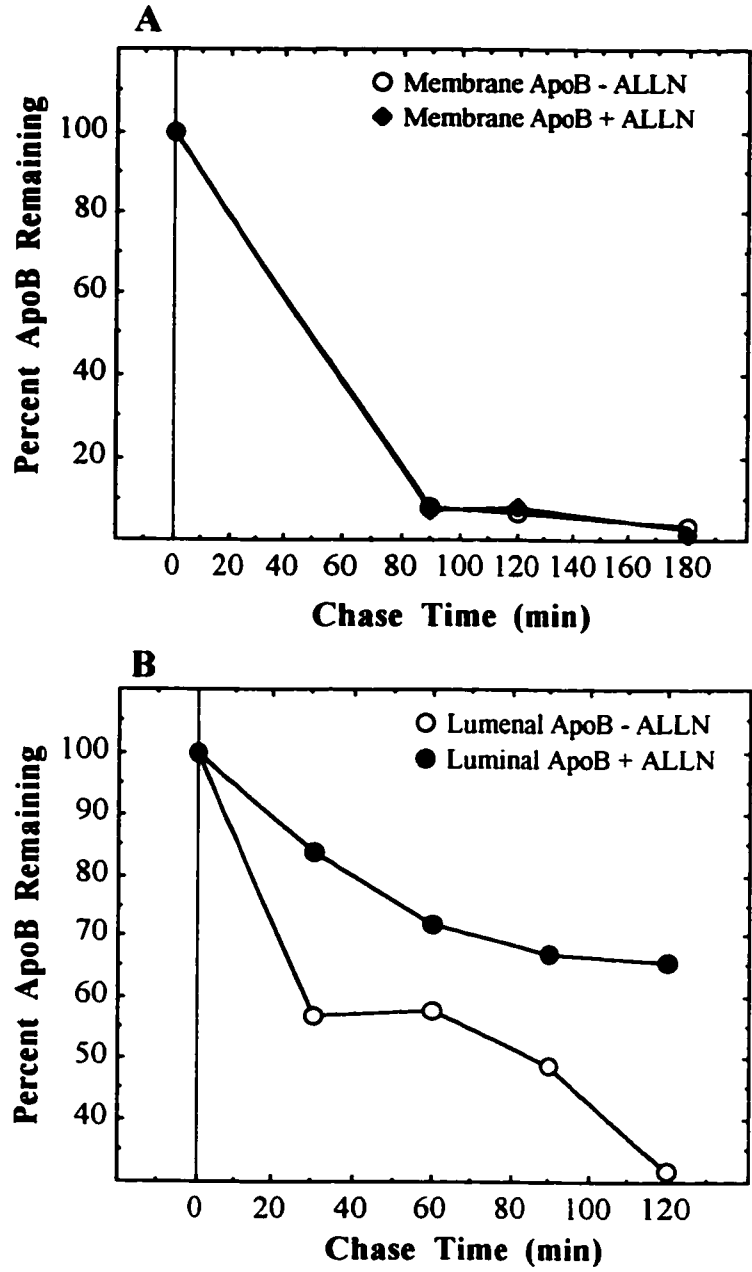


Figure 3.28**Effect of ALLN on Degradation of ApoB in the Membrane and Lumen of ER in HepG2 Cells****Legend**

HepG2 cells were pulsed, chased, permeabilized, and incubated in CSK in the absence or presence of ALLN (40 $\mu\text{g/mL}$). Cells were then solubilized and microsomal membrane (A) and luminal (B) fractions were separated by ultracentrifugation as described in Materials and Methods. ApoB was then immunoprecipitated from membrane (A) and luminal (B) fractions. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. The bands corresponding to intact apoB and the 70 kDa fragment were cut out of the gels and their radioactivity was counted as described in Materials and Methods. Percent apoB remaining was calculated based on the amount of intact apoB present at 0h.

Figure 3.28



CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 *In vitro* Translation of ApoB mRNA in a mRNA-Dependent HepG2 Cell-Free System

Due to its potentially positive role in the development of atherosclerosis, understanding the nature of the mechanism(s) involved in overproduction of LDL apoB is of great biochemical and clinical importance. Evidence obtained by several laboratories indicate that apoB mRNA levels do not change in response to acute stimuli. Various metabolic states, such as fasting and carbohydrate overload, which alter apoB secretion, do not affect apoB mRNA levels (Davis *et al.*, 1989a; Lusis *et al.*, 1987). Free fatty acids, such as oleate and butyrate which stimulate, or insulin which suppresses apoB secretion also exert their effect without changing apoB mRNA levels (Pullinger *et al.*, 1989; Dashti *et al.*, 1989; Moberly *et al.*, 1990; Kaptein *et al.*, 1991). These observations have focused attention on the co- and post-translational mechanisms that may regulate apoB production. Modulation of apoB mRNA translation might be one of the regulatory points in apoB production. *In vitro* translation of apoB mRNA could provide important information about factors regulating apoB production at this step. Molecular events involved in the segregation and processing of secretory proteins into the ER can also be studied by using a coupled *in vitro* translation/translocation system. Cell-free systems have been prepared from many eukaryotic cells and used for *in vitro* translation and/ or translocation studies. Some examples include cell-free systems from rabbit reticulocytes

(Pelham and Jackson, 1976, Jackson and Hunt 1983), Erlich ascites tumor cells (Henshaw and Panniers, 1983), mouse and rat liver cells (Eisenstein and Harper, 1984; Morley and Jackson, 1985), wheat germ cells (Tse and Taylor, 1977), and xenopus oocytes (Matthews and Colman, 1991). Because of the complexity and length of the apoB100 molecule (550 kDa), it has been difficult to demonstrate the translation of its mRNA (14 kb) *in vitro*. Attempts to use cell free systems for *in vitro* synthesis of full-length apoB have not been successful (Olofsson *et al.*, 1985; Bostrom *et al.*, 1984; Reuben *et al.*, 1988). Bostrom *et al.* (1984) showed the synthesis of an 80 kDa polypeptide antigenically related to apoB100 in reticulocyte lysate. Studies with reticulocyte lysate in our laboratory also showed the synthesis of a series of polypeptides ranging in size from 125 kDa to 170 kDa upon translation of apoB mRNA (Theriault, 1994). These results suggest that the difficulty in the synthesis of full length apoB may be due to problems associated with isolating full-length apoB mRNA or in the inherent inability of a heterologous cell-free system such as reticulocyte lysate to sustain the synthesis of the entire apoB molecule.

A cell-free system from HepG2 cells was recently prepared in our laboratory which uses endogenous mRNA to synthesize liver specific proteins including apoB100. This system allowed us to study the regulatory effects of insulin (Adeli and Theriault, 1992) and thyroid hormone (Theriault, *et al.* 1992) on apoB gene expression. Treatment of the lysate with edeine and polyvinyl sulfate showed that the synthesis of a significant proportion of the newly synthesized proteins was due to initiation of mRNA translation in the lysate. A major drawback of this system, However, was that it could not be used for

translation of exogenous mRNAs (Adeli and Theriault, 1992). To obtain a better knowledge of the mechanisms involved in the initiation of translation of apoB mRNA efforts have been made to make the above cell-free system mRNA dependent, and sensitive to exogenous mRNA.

Micrococcal nuclease (ribonuclease (deoxyribonuclease) 3'-nucleotidohydrolase) was used to hydrolyze the endogenous RNA. This enzyme is an extracellular phosphodiesterase purified from *staphylococcus aureus* (or *Micrococcus pyogenes*) and is able to hydrolyze either RNA or DNA producing 3'-phosphomononucleotides and dinucleotides (Reddi 1958; Reddi 1960). Endogenous mRNA was destroyed by incubating the HepG2 cell lysate with micrococcal nuclease. To function, the enzyme requires Ca^{2+} . The concentration of Ca^{2+} necessary depends on the pH of the reaction medium (Cuatrecasas *et al.*, 1967). Following the hydrolysis reaction, the enzyme can be totally inactivated by chelating calcium ions with EGTA. In this study EGTA with a final concentration of 2 mM was used to inhibit the enzyme. It has been shown that EGTA at this concentration does not affect protein synthesis rate (Giannakouros and Georgatsos, 1988).

The mRNA-dependent lysate prepared from HepG2 cells proved to be very active towards the translation of exogenous mRNA as demonstrated by the translation of both homologous (HepG2 RNA) and heterologous (Brome Mosaic Virus RNA) mRNAs. Based on the radioactivity counts from several experiments, the minimum efficiency obtained for the translation of BMV RNA and poly A⁺ RNA, extracted from HepG2 cell

were more than 50 and 40% of the intact lysate, respectively. Supplementing the lysate with cytoplasmic RNA extracted from HepG2 cells, resulted in the synthesis of wide spectrum of liver specific proteins including full length albumin and apoB as evidenced by immunoprecipitation of these proteins from translation products. Unlike other common mRNA dependent cell-free lysates, addition of tRNA or postribosomal supernatant did not result in further stimulation of the translation of poly A⁺ RNA or cytoplasmic RNA (data not shown). This is probably an indication of the presence of all the required factors in the lysate for the synthesis of a wide range of proteins including proteins as large as apoB100 without the need for the addition of any exogenous factor to help the translation reaction. The mRNA-dependent HepG2 lysate could serve as a useful system to study mechanisms regulating the initiation and elongation of the translation of apoB100 mRNA which could in turn lead to a better understanding of the role of potential translational factors involved in the overexpression of apoB under certain hormonal or metabolic states.

Important understanding of the early events in protein synthesis and secretion have been acquired by the use of *in vitro* translation and translocation systems (Walter and Lingappa, 1986). The initial stages of protein segregation into the endoplasmic reticulum can be reconstructed in a cell-free translation system by adding components necessary for translocation. More often than not, in most assays reported (Walter and Blobel, 1983), the components added are not derived from the same source or species. A common approach to achieve co-translational translocation of *in vitro* translated proteins is to add dog pancreas microsomes to the translation system. Such heterologous systems

are not always compatible. For example, the wheat germ lysate but not the reticulocyte lysate was found to be inhibited by dog pancreas microsomes (Shields and Blobel, 1978). Similarly, the *Saccharomyces cerevisiae* pre-proalpha factor can be segregated into yeast microsomes only if it is synthesized in yeast lysate, whereas the same protein made in wheat-germ lysate will not enter such membranes (Hansen *et al.*, 1986). Clearly, this has complicated interpretation of the results from these heterologous systems, and has raised many doubts about the validity of these systems. Obviously, if one is to examine events taking place in the living cell, components must be of the same origin. Indeed, the development of homologous translation-translocation systems is crucial to our understanding of these early secretory events. Several such systems have been developed, but none with human liver cells (Walter and Blobel, 1983). The HepG2 translation/translocation system reported here should prove useful in studying the *in vitro* expression of not only apolipoproteins but also other liver-specific proteins. The HepG2 lysate is simple to prepare and contains all the essential factors for both mRNA translation and protein translocation. Work in our laboratory (Theriault, 1994) has shown that the HepG2 lysate contains significant amounts of microsomal vesicles as evidenced by ultracentrifugation and the high NADPH-cytochrome c reductase activity present in the lysate. The microsomal membranes present were found to be intact as assessed by protease protection assays. The presence of endogenous microsomes eliminates the need to supplement the translation system with exogenously added microsomes, which are difficult to prepare and are very costly.

The mechanisms involved in the translocation of apoB across the ER membrane has recently attracted much attention. It has been suggested that regulation of the rate of apoB translocation may control the rate of apoB secretion (Davis *et al.*, 1990a; Davis *et al.*, 1989b; Du *et al.*, 1994; Thrift *et al.*, Rusinol and Vance, 1995; Bonnardel and Davis, 1995). ApoB chains that are successfully translocated across the ER are used to assemble secretion-competent lipoprotein particles, whereas those that fail to fully translocate are diverted to degradation (Davis *et al.*, 1990a; Boren *et al.*, 1992; Chuck *et al.*, 1990; Pease *et al.*, 1991). To study apoB translocation, most *in vitro* studies have used traditional cell-free translation systems supplemented with dog microsomes. These studies were performed with truncated forms of apoB as these translation systems are incapable of synthesizing full-length apoB (Chuck *et al.*, 1990; Pease *et al.*, 1991; Chuck and Lingappa, 1993). The use of dog pancreas microsomes also raises the concern that these microsomes may be functionally different than hepatic microsomes, and may lack the essential factors that facilitate translocation of apoB. The HepG2 *in vitro* translation/translocation system reported here offers a novel homologous model system which could be useful for future investigations into the mechanisms of apoB translocation and possible regulation of this process. It may also help to settle the current controversy as to the role of a unique translocation system in controlling apoB biogenesis.

4.2 Modulation of ApoB Secretion and Degradation by Hypolipidemic Drugs

4.2.1 Effect of Nicotinic Acid, Probucol, and Fibric Acid Derivatives

Nicotinic acid, has been used as a hypolipidemic drug since the time of its discovery in 1955 (Altschul *et al.*, 1955). Pharmacological doses of nicotinic acid are

useful in the treatment of most forms of hyperlipoproteinemia (Brown and Goldstein, 1990). It has been known for several years that nicotinic acid decreases VLDL production (Grundy *et al.*, 1981). Apart from its effects on lipoprotein cholesterol, nicotinic acid is also fibrinolytic and a vasodilator agent (Weiner and van Eys, 1983). These effects of niacin are probably mediated by the release of the endogenous prostacyclin PGI₂ (Gryglewski *et al.*, 1988). The vasodilating function of nicotinic acid, However, is short and acute tolerance to subsequent doses develops rapidly (Weiner and van Eys, 1983). At doses lower than the pharmacological dose, nicotinic acid increases plasma HDL cholesterol level by about 30% without changing the total cholesterol and triglycerides significantly (Luria, 1990). However, the mechanism of nicotinic acid function in lowering VLDL cholesterol is not known yet. Inhibition of lipolysis in adipose tissue, decreased estrification of triglycerides in the liver and increased activity of lipoprotein lipase have been suggested as possible mechanisms of its function (Gey and Carlson, 1971). In the present study, the effect of niacin on apoB degradation in HepG2 cells which could result in decreased production of VLDL particles was investigated. Results regarding the effect of nicotinic acid on apoB degradation do not show a significant difference between control and niacin treated cells, therefore ruling out the possibility of stimulation of apoB degradation by niacin.

The effect of probucol on the reduction of xanthomas in homozygous patients with familial hypercholesterolemia and also of athromas in WHHL rabbits has been attributed to the inhibition of LDL oxidation by probucol. LDL isolated from WHHL rabbits treated with probucol is resistant to oxidation by cupric ions (Kita *et al.*, 1987:

Steinberg *et al.*, 1989). In this study we investigated the effect of probucol on apoB degradation in a permeabilized HepG2 system. ApoB remaining in probucol treated cells did not show any significant difference to that of the control cells, indicating that probucol does not have any effect on apoB degradation in HepG2 cells. This is in agreement with previous published work showing probucol does not cause a significant change in the rate of either synthesis or catabolism of LDL (Atmeh *et al.*, 1983).

Among the fibric acid derivatives used in this study, clofibrate (200 µg/mL) and gemfibrozil (120 µg/mL) showed a reduction in apoB remaining which was significantly different from that of control cells, indicating the possibility of stimulation of apoB degradation. Immunoprecipitation of albumin in further experiments, However, revealed that the decrease was not unique to apoB and protein synthesis in general was inhibited in drug treated cells. Since the growth of drug treated cells was also much slower than the control cells, the observed effect on apoB is probably due to toxicity of the drugs to the cells rather than inhibition of apoB synthesis or stimulation of its degradation.

4.2.2 Effect of Atorvastatin

Numerous evidence suggest that once synthesized, apoB is subjected to posttranslational regulation, a process which is closely linked to the lipid status of the cell. The supply of one or more of the core lipids (triglycerides and cholesteryl ester) in apoB-containing lipoproteins plays an important role in regulating the assembly and secretion of these lipoproteins. Human liver can apparently "vary the type and quantity of apoB particles secreted in response to the load and type of lipid it has received" (Sniderman and Cianflone, 1993). Increased delivery of either fatty acids, sterols, or both

to the liver may result in overproduction of apoB particles (Sniderman and Cianflone, 1993). The amount of newly-synthesized apoB used for secretion may be modulated by the available supply of triglycerides (Dixon *et al.*, 1991; Wu *et al.*, 1994a; Boren *et al.*, 1993b; Wu *et al.*, 1994) cholesteryl ester (Kohen-Avramoglu *et al.*, 1995; Cianflone *et al.*, 1990; Dashti 1992), or possibly specific pools of phospholipids (Vance and Vance, 1990). Some studies have suggested that increased synthesis of triglyceride may upregulate apoB secretion by increasing the recruitment of the ER-translocated apoB to form mature lipoproteins (Boren *et al.*, 1993b; Wu *et al.*, 1994) while in other studies increased cholesteryl ester synthesis rather than triglyceride synthesis has been suggested as the immediate regulator of apoB secretion (Kohen-Avramoglu *et al.*, 1995; Cianflone *et al.*, 1990; White *et al.*, 1992). Overall, these studies suggest that apoB may be made in surplus and its secretion rate may be decided by the availability of the intracellular lipid supply.

Intracellular lipid pools most likely regulate apoB production via posttranslational mechanisms which may involve facilitating the translocation of newly synthesized apoB across the ER membrane, reducing apoB degradation and/or enhancing its assembly into secretion-competent lipoprotein particles. In the present study atorvastatin, a new HMG-CoA reductase inhibitor with potent inhibitory effects on the intracellular rate of cholesterol synthesis, was used to investigate the effect of inhibition of cholesterol synthesis on apoB translocation into the ER and its intracellular degradation. Atorvastatin has been shown to decrease cholesterol synthesis to a considerable extent *in vitro*, *ex vivo*, and *in vivo* (Shaw *et al.*, 1990; Krause and Newton, 1991; Bocan *et al.*, 1992, Bocan *et*

al., 1994). Results obtained in our laboratory also demonstrated that treatment of HepG2 cells with atorvastatin under basal or lipid rich conditions results in a significant reduction in apoB secretion. Several previous studies have been performed to investigate the effect of HMG-CoA reductase inhibitors on apoB secretion with mostly conflicting results. Ribeiro *et al.* (1991) studied the effect of simvastatin on primary cultures of rat hepatocytes and reported an stimulation of apoB secretion. On the other hand, Sato *et al.* (1990) found that treatment of HepG2 cells by CS-514 did not influence the synthesis and secretion of apoB. We also found that lovastatin did not exert any effect on the degradation or production rate of apoB in HepG2 cells.

Previous studies have mostly focused on the effect of HMG-CoA reductase inhibitors on the secretion of apoB and have not explored the intracellular mechanisms by which these drugs may exert their effects on the production of apoB containing lipoproteins. To further investigate the mechanism of atorvastatin action on apoB secretion, the semipermeable HepG2 system developed recently in our laboratory (Adeli, 1994) was used. Results from these experiments showed that atorvastatin decreased apoB secretion by stimulating its degradation in HepG2 cells. Further investigation showed that both membrane bound and luminal apoB pools were affected by the drug and there was a higher level of degradation in both pools under drug treated conditions. Atorvastatin showed its stimulatory effect on apoB degradation under basal as well as oleate and LDL treated conditions.

Results regarding the effect of atorvastatin on the translocation of apoB are consistent with the current research suggesting that translocation may be a key regulatory

point in apoB secretion (Davis *et al.*, 1989; Davis *et al.*, 1990; Du *et al.*, 1994; Rusinol and Vance, 1995; Bonnardel and Davis, 1995). There is considerable evidence to suggest the existence of two distinct pools of intracellular apoB. A luminal (trypsin-resistant) apoB pool which is utilized for the assembly of lipoproteins and a membrane-bound (trypsin-susceptible) apoB pool which is thought to be shunted to a degradative pathway (Davis *et al.*, 1990; Dixon *et al.*, 1991; Bonnardel and Davis, 1995). Data obtained in this study demonstrates that atorvastatin reduces the amount of apoB that is translocated into the lumen of the ER and increases the proportion that becomes membrane-bound. The reduced translocation may be the result of the depletion of cholesterol from HepG2 cells. This depletion of lipid may reduce the number of apoB containing lipoprotein particles and increase the amount of superfluous apoB. A number of studies have demonstrated the importance of lipid availability on the translocation apoB (Boren *et al.*, 1990; Boren *et al.*, 1992). The presence of oleate has been suggested to facilitate the translocation of apoB into the lumen and thereby reduce the percentage of apoB that becomes prone to degradation (Dixon *et al.*, 1991).

Although the primary mode of action of atorvastatin with respect to reduced apoB translocation and degradation may be the availability of cholesterol, the present data suggests that atorvastatin may also have a direct effect on apoB production. The results demonstrating an atorvastatin-mediated reduction in the percentage of apoB translocated and an increase in its degradation in LDL-treated cells were somewhat surprising. Intuitively, one might expect that an exogenous source of cholesterol might negate the effects of atorvastatin with respect to apoB translocation and degradation. The possibility

exists that atorvastatin may directly impair the translocation of apoB across the ER membrane, and this effect may be independent of lipid availability. The atorvastatin-mediated stimulation of apoB degradation in LDL-treated HepG2 cells may be the end result of the impaired translocation of apoB. Evidence that the actual process of apoB translocation across the ER may be a crucial regulatory point in the production of lipoproteins has recently been suggested by Rusinol and Vance (Rusinol and Vance, 1995). They have shown that the supplementation of primary rat hepatocytes with phosphatidylmonomethylethanolamine decreased the secretion of apoB. This decrease was attributed to a decrease in the translocation of apoB and was independent of lipid availability. Furthermore, Davis and co-workers (Bonnard and Davis, 1995) recently used HepG2 cells to demonstrate that apoB translocation rather than degradation may be the primary mechanism that regulated apoB secretion. Recent data from our laboratory (manuscript in preparation) has also shown that the rate of apoB translocation can be modulated by altering the conformation of the nascent protein with agents such as DTT and cyclosporine.

The data regarding the atorvastatin-mediated impairment of apoB translocations is corroborated by the results of subcellular fractionation experiments performed in this study. When the ER membrane and luminal fractions were separated, it was found that the amount of apoB present in the membrane fraction of drug-treated cells was significantly higher than the apoB present on the membrane of control cells. This could be interpreted as an indication of defective translocation of apoB across the ER membrane caused by atorvastatin. Since it has already been shown that agents such as

phosphatidylmonoethylethanolamine are capable of impairing apoB translocation across the ER membrane (Rusinol *et al.*, 1993), it is possible that by integrating into the ER membrane, atorvastatin also decreases apoB translocation into the ER lumen. An interesting observation was that the stability of the membrane associated apoB was significantly decreased in atorvastatin treated cells compared to that of control cells. While only speculative at this point it is possible that atorvastatin may cause apoB to be abnormally integrated into the membrane such that it becomes more susceptible to degradation. The increased degradation may be the result of an altered conformation of the membrane-associated apoB which makes it is more accessible to proteolysis.

Intracellular degradation of the luminal fraction of apoB was also affected to the same extent by atorvastatin. The data showing that HepG2 cells treated with atorvastatin contained a significantly lower amount of luminal apoB-containing lipoproteins further supports the suggestion that atorvastatin may be causing newly synthesized apoB to be shunted to the membrane-bound degradative pool. Fractionation of luminal apoB into the dense (HDL-like), and light (LDL-VLDL like) particles (Boren *et al.*, 1990; Boren *et al.*, 1992) showed that both fractions undergo a higher level of degradation in the presence of atorvastatin. However, the HDL-like particles showed a more profound susceptibility to degradation under atorvastatin treatment conditions. This effect could be exerted directly by inhibiting cholesterol synthesis and diminishing its accessibility for lipoprotein assembly, or by changing the composition and structure of nascent dense (HDL-like) lipoprotein particles. The exact mechanism(s) for the effect of atorvastatin on apoB degradation remains to be elucidated through further investigations.

Overall the results obtained further support the clinical evidence that atorvastatin decreases the production of LDL-apoB. It appears that this effect may be occurring at several levels. Atorvastatin has been shown to decrease the translocation of apoB into the lumen and increase its rate of degradation. This increased degradation may be the result of the impaired translocation. In addition, membrane-associated apoB is destabilized in the presence of atorvastatin. Taken together the data suggest that atorvastatin may decrease plasma LDL-apoB not only through upregulation of LDL receptors, but also through a decrease in the hepatic production of apoB containing lipoproteins.

4.3 Mechanisms of Intracellular ApoB Degradation

A digitonin-permeabilized cell system was developed from HepG2 cells in our laboratory (Adeli, 1994) which was used to partially characterize the intracellular degradation pathway for apoB. Results indicated that apoB degradation in permeabilized HepG2 cells occurs to the same extent as in the intact cells, is temperature and pH sensitive and can be stimulated by ATP. It was also shown that the apoB degrading system is localized in a pre-Golgi compartment and could be inhibited by ALLN (Adeli, 1994). Since apoB exists in the ER as membrane bound and luminal pools (Boren, 1992), it was not clear whether the results obtained represent both apoB species or not. In this study the permeabilized HepG2 system was employed to further investigate the intracellular localization of apoB degradation in HepG2 cells. The results indicated that apoB100 was degraded both when associated with the membrane and when present on lipoproteins in the secretory pathway. The degradation appeared to be a specific process for apoB100 since neither albumin nor α_1 -antitrypsin showed any significant degradation

under the same condition. The observation that albumin is not degraded under these experimental conditions is in agreement with previous results (Adeli, 1994). Also the observation that the membrane associated form of apoB100 is sorted to degradation supports previous results from our lab and other groups (Boren *et al.*, 1992; Thrift *et al.*, 1992; Cartwright *et al.*, 1993). However, this study was the first to demonstrate that apoB100 can be subjected to posttranslational degradation also when present in the secretory pathway.

It has been shown that the protease inhibitor ALLN (Thrift *et al.*, 1992; Adeli, 1994) inhibits the degradation of apoB and promotes the secretion of the protein (Adeli, 1994; Sakata *et al.*, 1993). Previous studies in our laboratory on permeabilized Hep G2 cells (Adeli, 1994) showed that ALLN is the most potent inhibitor of apoB degradation with a dose- dependent effect on this degradation. It was found very effective in abolishing the degradation of apoB and stopping the generation of apoB degradation fragments. The current results indicate that ALLN inhibited only degradation of apoB100 that was present on lipoproteins in the lumen of the secretory pathway.

The intracellular site at which the apoB degradation occurs is still poorly understood. Most studies point to the ER or a closely associated compartment (Davis *et al.*, 1989; Davis *et al.*, 1990; Furukawa *et al.*, 1992; Adeli, 1994), which is in agreement with the degradation site of other secretory proteins (Lippincott-Schwartz *et al.*, 1988; Klausner and Sitia, 1990, 1993). However, three studies have reported post ER or Golgi degradation of apoB in primary rat hepatocytes (Wang *et al.*, 1995; Sparks and Sparks, 1993; Verkade *et al.*, 1993). Recent studies on the degradation of other secretory proteins

suggest the possible involvement of an intermediate compartment. Intracellular degradation of the major histocompatible complex I molecules was recently shown to occur in a tubular system between ER and Golgi (a post ER, pre Golgi compartment) (Raposo *et al.*, 1995). Interestingly both ubiquitin and its associated protease E1 were associated with this compartment, suggesting the possible involvement of a ubiquitin-mediated degradation pathway (Raposo *et al.*, 1995).

The protease involved in apoB degradation might be similar to the protease responsible for the degradation of other proteins such as HMG-CoA reductase, apoE and IgM, which also respond to ALLN. ALLN is a potent inhibitor of calcium-dependent cysteine proteases and lysosomal cathepsins L and B. Since the degradation of apoB is inhibited by ALLN but not lysomotropic agents such as leupeptin and NH₄Cl, it is possible that calpain-like proteases are involved in the ER degradation of apoB. However, the pH activity of calpains is more basic than that observed for the apoB degradation system. A recently discovered ER cysteine protease, called ER60 which is inhibited by ALLN (Urade *et al.*, 1992) may be a better candidate for the protease involved in the ER degradation of apoB and possibly other ER localized proteins. Interestingly, the ER-60 protease was recently shown to be inhibited by negatively charged, acidic phospholipids, a finding that may have implications in lipoprotein assembly. A second protease, ERp 72 with similar characteristics has also been characterized (Otsu *et al.*, 1995). The ER60 protease appears to associate with protein disulfide dismutase and degrade misfolded human lysozyme (Otsu *et al.*, 1995), suggesting that this protease may be a component of the pre-Golgi degradation system.

The posttranslational degradation is of potential importance in the regulation of apoB100 secretion. Previous results (Boren *et al.*, 1993) may indicate that apoB100 that is assembled into a mature lipoprotein is degraded. Other observations (Sakata *et al.*, 1993; Wu *et al.*, 1994; Ginsberg, 1995) may support a more regulatory role for degradation in the process of apoB100 secretion. Characteristic for this regulatory degradation is that it is inhibited by ALLN (Thrift *et al.*, 1992; Adeli, 1994; Sakata *et al.*, 1993) and it appears to generate rather specific proteolytic intermediates such as a 70 and a 85 kDa protein. Results obtained in this study may indicate that the regulated degradation occurs at least to a certain degree on lipoproteins in the secretory pathway. In addition to the inhibition of the degradation with ALLN, the results also indicate that the apoB100 fragments, including the recently described 70 kDa protein, appear in association with the lipoproteins in the lumen of the secretory pathway. The generation of these fragments was completely inhibited by ALLN. This clearly indicates that the generation of the luminal apoB fragments is ALLN-sensitive and further confirms that luminal degradation of apoB is inhibited by ALLN.

In contrast the degradation of the membrane associated apoB was not sensitive to ALLN. However, a small amount of the 70 kDa fragment was detected in the membrane pellet after the carbonate extraction. The levels appeared to be significantly lower than those found in the luminal content. The reason for this could simply be that this fragment is only relatively weakly associated with the lipids and therefore is extracted with sodium carbonate thus ending up in this extract together with the lipoproteins. It should. However, be kept in mind, that small amounts of secretory proteins, that are present in the

ER lumen, will associate with the membrane during the carbonate extraction (Figure 3.23 and Bostrom *et al.*, 1986). Thus a portion of apoB100 as well as of the 70 kDa fragment that are associated with the microsomal membrane could be expected to represent a non-specific association of material from the lumen. Moreover, the observation that ALLN clearly inhibits the appearance of the 70 kDa fragment while it does not interfere with the degradation of the membrane associated apoB100, makes it less likely that this fragment is derived from degraded membrane associated apoB100.

When evaluating the physiological importance of the observation that there is a difference in the degradation of apoB100 in the lumen and the membrane. it should be kept in mind that we are dealing with cells that have been permeabilized and lack the cytosol. Since several studies have indicated that membrane associated apoB100 expose sequences on the cytosolic side of the ER membrane, it is possible that proteins that are present in the cytosol are of importance for the degradation of this pool of apoB100. In favor of this possibility is the recent observation that membrane associated apoB100 is protected by ALLN not by the inhibition of a protease but rather by an induction of hsp70 which interacts with the cytosolic part of apoB100 and protects it from degradation (Zhou *et al.*, 1995). Obviously such a mechanism would be masked in permeabilized cells.

The results presented in this study, i.e., the difference in kinetics as well as the difference in sensitivity to ALLN indicate, however, that in Hep G2 cells, two different pathways exist for the degradation of apoB100. One that is specific for the assembled lipoproteins in the lumen of the secretory pathway and one that may have the membrane associated protein as its target. The results also demonstrate that the luminal apoB100 is

degraded via an ALLN-sensitive pathway that generates the types of degradation intermediates that previously have been suggested to be associated with the regulatory posttranslational degradation.

4.4 Conclusion

The mRNA-dependent cell-free system prepared from HepG2 cells was shown to be very active towards the translation of exogenous mRNA. Both homologous (HepG2) and heterologous (Brome Mosaic Virus) mRNAs were efficiently translated in this system. SDS-PAGE analysis of translation products revealed that the lysate is capable of synthesis of full-length apoB100, a 550 kDa protein. Addition of exogenous tRNA or postribosomal supernatant did not improve the translational efficiency in the lysate, suggesting that the lysate prepared from HepG2 cells contains all the necessary factors to sustain translation of exogenous mRNAs. There was However, a positive correlation between translational efficiency and concentration of labeled methionine and incubation time. Translational efficiency of the system also depends on the concentration of Mg^{2-} and K^{+} ions in the lysate.

Studies on the effect of hypolipidemic drugs on the degradation of apoB100 in permeabilized HepG2 cells showed that among the nicotinic acid, probucol, and fibrate acid derivatives (clofibrate, bezafibrate, and gemfibrozil), clofibrate effectively stimulated apoB degradation. The other drugs However, did not affect apoB degradation. The newly developed HMG-CoA reductase inhibitor, atorvastatin also decreased stability of apoB100 in HepG2 cells and stimulated its degradation. Atorvastatin showed its effect under both lipid rich and normal conditions. Fractionation of different apoB pools in the

ER of HepG2 cells showed that atorvastatin stimulates degradation of both membrane bound and luminal apoB. Atorvastatin also decreased translocation of apoB into the ER lumen. Separation of different apoB species in the lumen of ER by ultracentrifugation revealed that the HDL-like particles, containing apoB are more susceptible to degradation under the effect of atorvastatin compared to the mature LDL-VLDL like particles. Together these effects resulted in the reduction of apoB100 secretion by HepG2 cells.

Further studies on degradation of apoB100 pools in permeabilized HepG2 cells showed that both the apoB bound to the ER membrane and the apoB present as lipoproteins in the secretory pathway were degraded. Neither albumin nor α 1-antitrypsin showed any significant posttranslational intracellular degradation under the same conditions, suggesting the specificity of the apoB degrading system. The kinetics for the turnover of membrane bound apoB appeared to be different from that of the luminal apoB as it was shown that degradation of membrane bound apoB occurs at a faster rate. Moreover, while degradation of the luminal apoB100 was inhibited by ALLN, degradation of membrane bound apoB did not respond to ALLN. Together, these results suggest the possibility of the presence of different pathways for degradation of luminal and membrane bound apoB in the ER.

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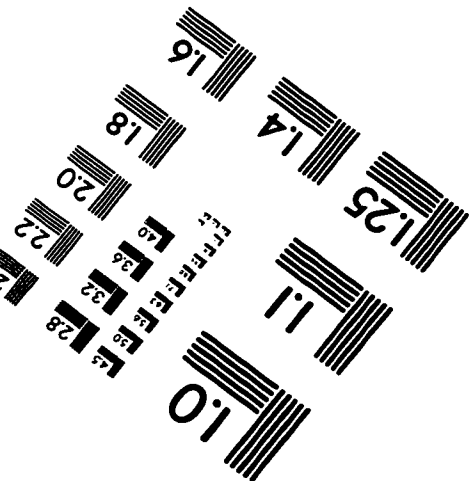
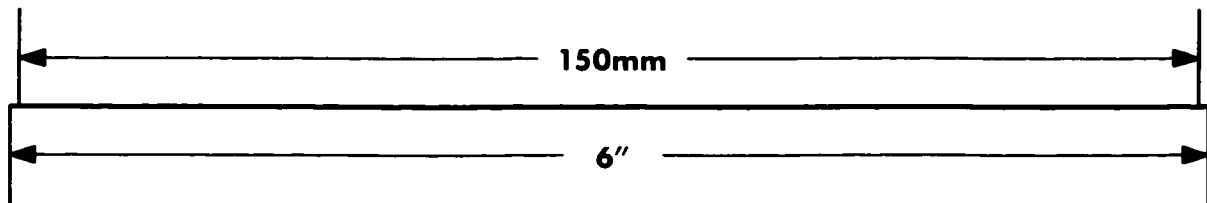
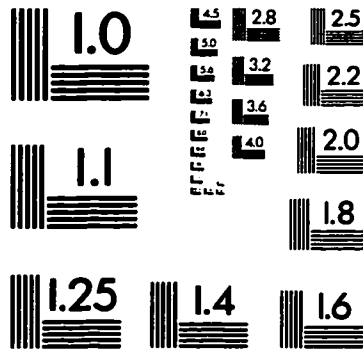
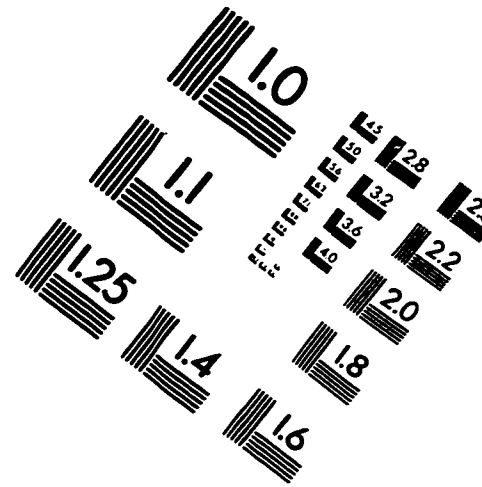
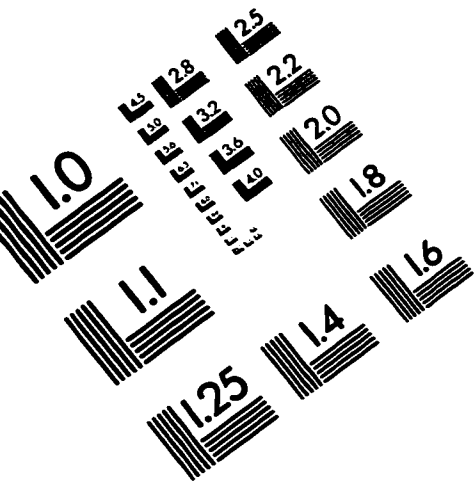
Effect of Hypolipidemic Drugs on Hepatic Degradation of Apolipoprotein B and Production of ApoB-Containing Lipoproteins. AACC meeting, May 1994, Frankenmuth, Michigan, U. S. A.

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